# VALIDATION OF LAB-ON-CHIP CAPILLARY ELECTROPHORESIS SYSTEMS FOR TOTAL RNA QUALITY AND QUANTITY CONTROL

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## ABSTRACT

*Purity and good RNA quality are important elements for the overall success of RNA based analysis methods like microarrays and real time qRT-PCR. There are two commercially available automated systems – the Experion (Bio-Rad Laboratories) and the 2100 Bioanalyzer (Agilent Technologies) – that provide both RNA sample quality and quantity analysis. In this study different aspects like the reproducibility and sensitivity of both systems were analyzed by determining the total RNA quality and quantity extracted from various bovine tissues. Regarding quantitation, the Experion is more sensitive than the 2100 Bioanalyzer. Both systems overstate the concentration by 19-29% compared to the photometric values. For RNA quality determination, both systems show highly comparable reproducibility. With the RNA integrity number (RIN) the 2100 Bioanalyzer offers an additional opportunity to quantify the RNA quality.*

**Keywords**: RNA integrity, RNA quality, RNA quantity, transcriptomics, lab-on-chip, capillary electrophoresis

## Introduction

Methods for Gene Expression measurements like microarray technology and quantitative real time RT-PCR (qRT-PCR) require high quality RNA (1, 3, 4, 5). The purity of RNA is normally assessed by its $OD_{260\ nm}/OD_{280\ nm}$ ratio (2) but using this tool no information is given according RNA integrity. For decades, scientists have gained essential data about their nucleic acid RNA samples from the use of agarose gel-based electrophoresis. Gel electrophoresis, the movement and separation of charged particles in response to an electric field, results in an unparalleled and irreproducible resolution of RNA molecules. However, the process is composed of a series of manual steps that require numerous pieces of equipment, various reagents, and several hours to gain the information needed from sample separations. Since this traditional process requires significant time and manual labour, automation has been a key desire of scientists. The Experion (Bio-Rad Laboratories, Hercules, CA) and the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) system apply innovative micro-fluidic separation technology to both automate and accelerate this process.

The lab-on-chip is an integrated part of the micro-fluidic system, working together with specialized computer-controlled instrumentation used to manipulate the timing and sequence of the processes designed into the chip. As a result, these systems inherently generate accurate and reproducible data. Nevertheless, chip design must provide optimal channel and sample well dimensions, and system design must provide precise control over temperature, flow rates, and sample injection and separation voltages, amongst good laboratory praxis, in order to produce optimal results. When micro-fluidic chips are used for electrophoresis, the process is very similar to that of traditional gel-based electrophoresis. The main difference is that micro-fluidics enables the miniaturization and combination of multiple steps of gel-based electrophoresis – separation, staining, containing, imaging, and even basic data analysis – into a single automated process.

In this study we determined the RNA quality and quantity of different bovine tissues by using the Experion (Bio-Rad Laboratories) and the 2100 Bioanalyzer (Agilent Technologies). The intention was to investigate the comparability and validity of the results delivered by both lab-on-chip systems. As the policy for these two devices does not differ, the analysis of all samples should lead to similar results. The appearance of differences in the achieved results has to be borne in mind when interpreting data from these two devices.

## Materials and Methods

### Total RNA

Total RNA purification was performed by an in-house standardized phenol-based extraction methods using TriFast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. Total RNA was extracted from various bovine tissues, and was carried out in RNAse-free environment. To show tissue independent quality and quantity studies, eleven different bovine tissue sources were under investigation. Purified total RNA was eluted in the column using RNAse-free water (Eppendorf, Hamburg, Germany). First RNA integrity was verified in triplicates by UV measurement, using the BioPhotometer (Eppendorf) and additionally the NanoDrop 1000 (Peqlab). Only samples with an $OD_{260\ nm}/OD_{280\ nm}$ absorption ratio higher than 1.85 were used for the further investigations.

### Hardware

For microcapillary electrophoresis measurement, the Experion system was used in conjunction with the Experion RNA

StdSens kit (Bio-Rad) and the 2100 Bioanalyzer with the RNA 6000 Nano LabChip analysis kit (Agilent Technologies) and the 6000 RNA ladder (Ambion, Austin, TX). Total RNA samples and ladders were prepared according to the protocols provided in the instruction manuals of the Experion RNA analysis kit and the Agilent RNA 6000 LabChip kit. To prevent systematic handling errors one single user performed the assays on both micro-capillary electrophoresis systems in parallel. In the project the comparability of the results of both analytical systems were analyzed with two different total RNA concentrations: 50 ng/µl or 200 ng/µl. The RNA Integrity Number (RIN) software algorithm of the 2100 Bioanalyzer permits the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact (6).

**Sample degradation**

The first sub-project was conducted concentrating on different RNA degradation levels. To get RNA samples with different degradation levels, but with the identical transcriptome and mRNA distribution, total cellular RNA was degraded by irradiation with ultraviolet (UV) light, as described earlier by Fleige et. al (5). Depending on the type of tissue each sample was placed under UV-lamp for a different period of time up to 1.5 h. Intact and degraded samples from identical tissue extraction, containing the identical transcriptome, were mixed in various ratios to generate a linear degradation gradient.

**Statistical analysis**

Descriptive statistics were generated using the Sigma Stat software, version 3.0 (SPSS Inc, Chicago, IL, USA). Mean, standard deviation (SD) and coefficient of variation (CV) between and within groups of samples were determined. Comparative statistical analyzes between groups were completed, using non-parametric statistical tests: Mann-Whitney Rank Sum Test. Coefficients were recorded when significant at a minimum of $p<0.05$. Higher significance levels were considered when available. Data were statistically processed in Sigma Plot 8.0 (SPSS, Inc.) and Excel (Microsoft, Seattle, US).

## Results and Discussion

In this study the RNA quality and quantity of different bovine tissues was determined by the Experion (Bio-Rad) and the 2100 Bioanalyzer (Agilent). By analyzing the same samples in parallel the comparability of both systems is given. By mixing an inter RNA sample with a qualitative degraded version of the same sample it was possible to impair the RNA quality factitiously (4). The creation of different mixing ratios made it possible to alter the samples in nuances from degraded to integer RNA quality. Therefore, it was possible to analyze the trend of the measurements of degraded RNA.

**Ribosomal 28S/18S ratio**

RNA integrity was first assessed by resolving the 28S and 18S ribosomal RNA band comparing both lab-on-chip systems. It is stated, that the ribosomal 28S/18S ratio plays an important role in determining the level of sample degradation in gel electrophoresis. Ratio analysis was done in total on 180 different total RNA sample profiles. Ratio data from 16 samples was not obtained due to device problems during the runs (8.9%). From one selected bovine tissue 12 RNA degradation levels were assessed using both separation systems. The results shown in figure 1 are from repeated determinations by using the Experion and a threefold determination with the 2100 Bioanalyzer. Furthermore, the Experion 28S/18S ratios are overall lower than the 2100 Bioanalyzer values. Both the 2100 Bioanalyzer and Experion data were also correlated to the RIN, because this mirrored RNA quality, notwithstanding this feature is not available in the Experion software. The graphs in figure 1 demonstrate the correlation between the ribosomal 28S/18S ratio and the degree of RNA degradation. As expected, the 28S/18S ratio rises with the increasing of sample quality. This applies to both systems. The comparison of the trend-lines leads to the conclusion that the slopes of the 2100 Bioanalyzer data are greater than those of the Experion. This trend is reflected in all regressions performed on various tissues (data not shown) and in selected single run comparisons, e.g. shown in figures 2a – 2c with total RNA extracted from bovine corpus luteum and caecum.

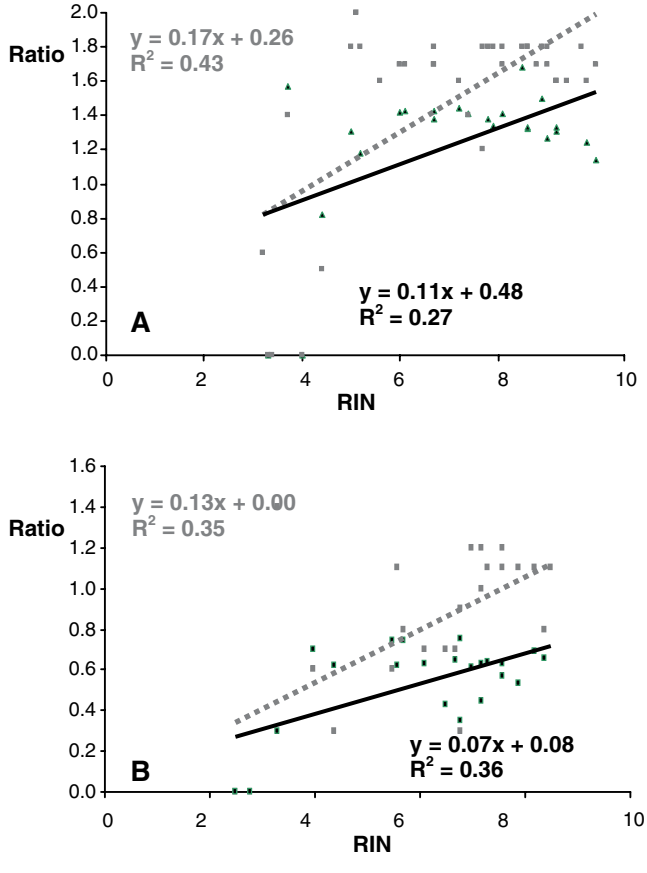
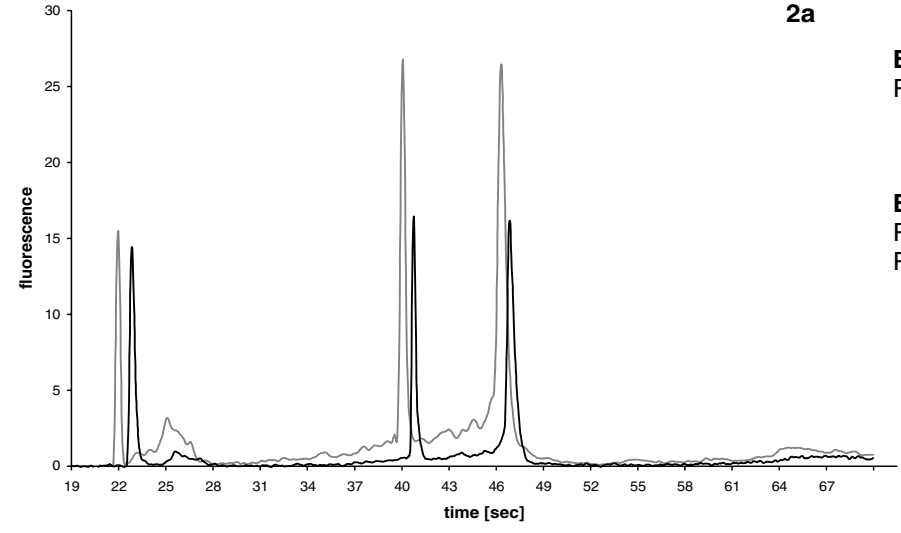
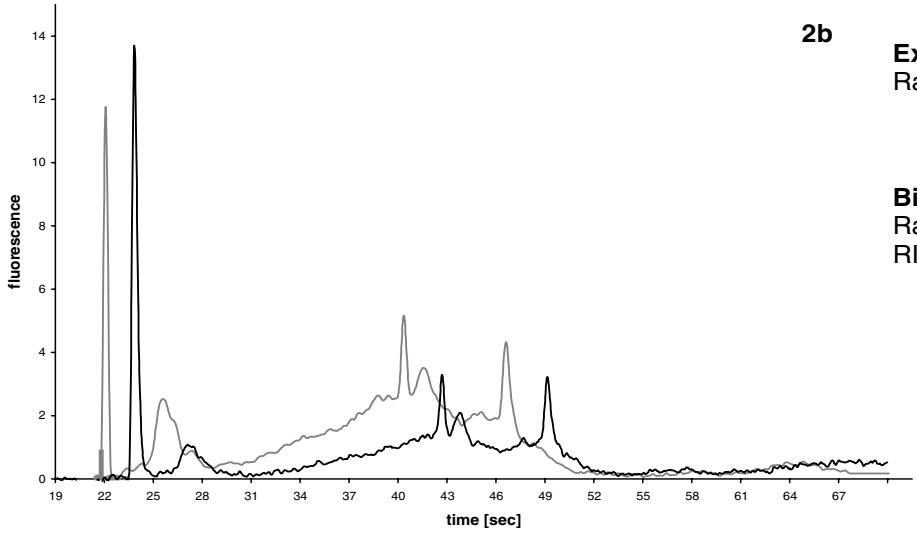



**Fig. 1.** Comparison of the 28S/18S ribosomal RNA ratios in Experion (solid line) and 2100 Bioanalyzer (dotted line). (A) 200 ng/µl input concentration (n = 36); (B) 50 ng/µl input concentration (n = 36)



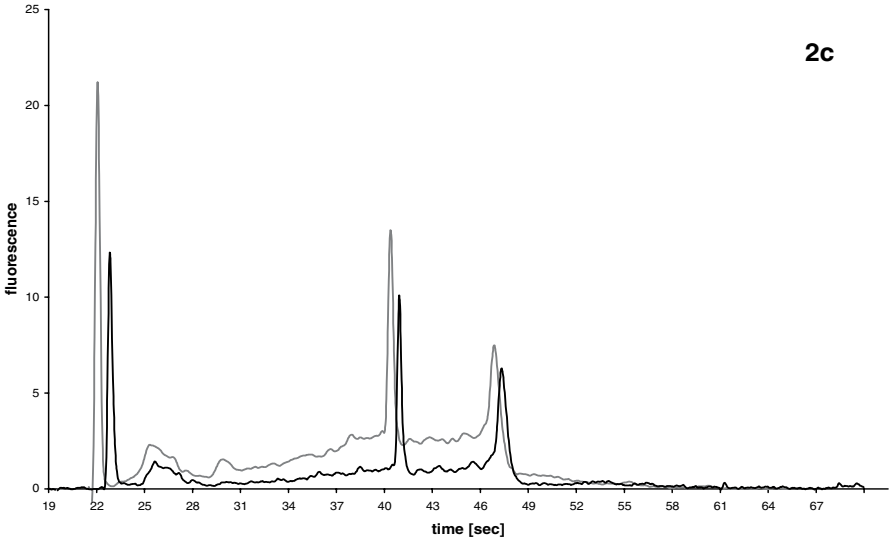

**Experion:** 130.31 [50.85 ng/µl]
Ratio [28S/18S]: **1.37**

**Bioanalyzer:** 44.8 [27.0 ng/µl]
Ratio [28S/18S]: **1.60**
RIN: 9.8




**Experion:** 130.31 [45.07 ng/µl]
Ratio [28S/18S]: **1.36**

**Bioanalyzer:** 44.8 [25.0 ng/µl]
Ratio [28S/18S]: **1.80**
RIN: 5.2




**Experion**: 165.34 [71.47 ng/µl]
Ratio [28S/18S]: **0.93**

**Bioanalyzer**: 63.3 [27.0 ng/µl]
Ratio [28S/18S]: **1.30**
RIN: 7.4

**Fig. 2a – 2c.** Comparisons of identical total RNA samples (50 ng/µl) which were assessed in both separation systems, in Experion (dotted line) and 2100 Bioanalyzer (solid line). Total RNA was extracted from bovine corpus luteum (**2a - 2b**) and bovine caecum (**2c**)

**TABLE 1**

Comparison of the gained area results

| System | Experion Ladder | 2100 Bioanalyzer Ladder |
|---|---|---|
| **Experion** (mean area units) | 400.66 | 496.92 |
| Deviation | 96.28 | 40.35 |
| CV [%] | 24.0 | 8.1 |
| **2100 Bioanalyzer** (mean area units) | 220.93 | 218.8 |
| Deviation | 18.78 | 52.29 |
| CV [%] | 8.5 | 24.2 |

The 28S/18S rRNA ratios increased with the rise of RNA quality more significant in the 2100 Bioanalyzer compared to the Experion (figure 1). The detailed analysis on both separation systems reveals that ribosomal 28S/18S ratio inadequately describes RNA integrity.

**Measured total RNA concentration**

In addition analysis for the concentration values was done on 724 RNA profiles with the low concentrated samples (50 ng/µl and well), where 31 profiles were not obtained due to device problems during the runs (4.3%). The system automatically calculated the RNA concentration, according to the initially standard curve. The distributions of the computed concentration values were noticeably different between both separation systems. The Experion quantification showed higher accuracy (figure 3), significant higher values ($p<0.001$), and more reproducible mean concentrations: 54.2 ng/µl (CV=39.1%), compared to the 2100 Bioanalyzer 43.4 ng/µl (CV=57.1%). Using 200 ng/µl total RNA per run, the analysis was performed on 80 RNA profiles in the Experion and 91 RNA profiles in the 2100 Bioanalyzer, where 8 samples failed (4.7%). The distribution of the computed concentration values were significantly different ($p=0.025$): mean 211.1 ng/µl (CV=14.7%) for Experion and 235.8 ng/µl (CV=27.4%) for the 2100 Bioanalyzer (figure 3).

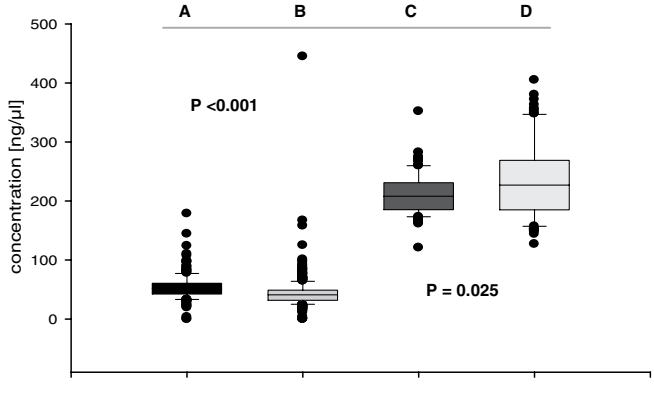



**Fig. 3.** Calculated concentration by both separation systems plotted in a box plot. (**A**) Experion 50 ng/µl (n = 198); (**B**) Bioanalyzer 50 ng/µl (n = 526); (**C**) Experion 200 ng/µl (n = 80); (**D**) Bioanalyzer 200 ng/µl (n = 91)

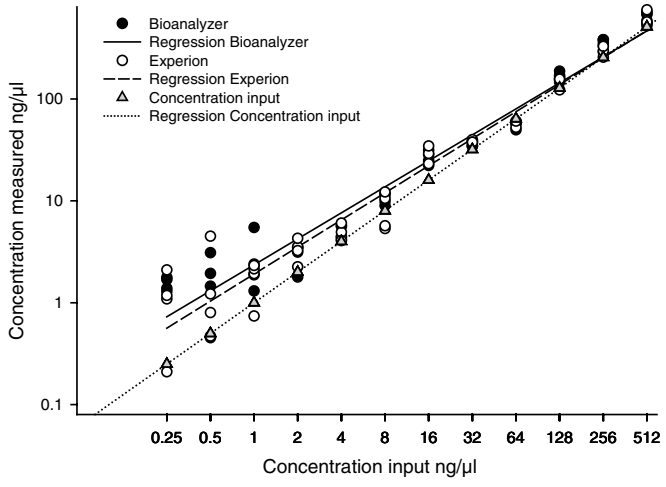



**Fig. 4.** Total RNA concentration measured by the Experion (dashed line) and the 2100 Bioanalyzer (solid line) versus total RNA input

**Sensitivity of both separation systems**

For testing the sensitivity a total RNA extract of bovine intestine was diluted to different concentration levels and then analyzed by both systems in parallel (n = 48). The concentrations per run were 512 ng/µl, 256 ng/µl, 128 ng/µl, 64 ng/µl, 32 ng/µl, 16 ng/µl, 8 ng/µl, 4 ng/µl, 2 ng/µl, 1 ng/µl, 0.5 ng/µl, 0.25 ng/µl. Regarding concentration measurements it can be observed that both platforms have high sensitivity down to 250 pg total RNA per run (figure 4), with high significant linearity ($p<0.001$) as shown by the linear regressions:

**Experion:** measured concentration = -1.404 + (1.190* input concentration); $r^2 = 0.953$

**Bioanalyzer:** measured concentration = -0.379 + (1.297* input concentration); $r^2 = 0.955$

But both systems overstate the concentrations determined in the BioPhotometer (Eppendorf) and the NanoDrop 1000 (Peqlab). Regression equantion show that the measurements done by the Experion are more close to the real input concentrations than those of the 2100 Bioanalyzer. The median overestimation of the Experion is 19.0% and that of the 2100 Bioanalyzer 29.7%, retrieved from the slope of the regression line.

**Sensitivity of the 28S/18S rRNA measurement**

Further the sensitivity of the 28S/18S rRNA measurement was determined in a serial dilution row as described above (512 ng/µl – 0.25 ng/µl, n=48). The Experion showed a decrease 28S/18S rRNA ratio correlating to lower RNA concentrations (**Fig. 5**). The fact that the 2100 Bioanalyzer graph shows an abrupt decrease corresponding to the concentrations lower than 8 ng/µl, which might be due its lower sensitivity. At concentrations lower than 1 ng/µl the 2100 Bioanalyzer does not measure any 28S/18S rRNA ratio. In both systems quantitation at higher concentrations is more exact what is visualized by the smaller error bars (**Fig. 5**).

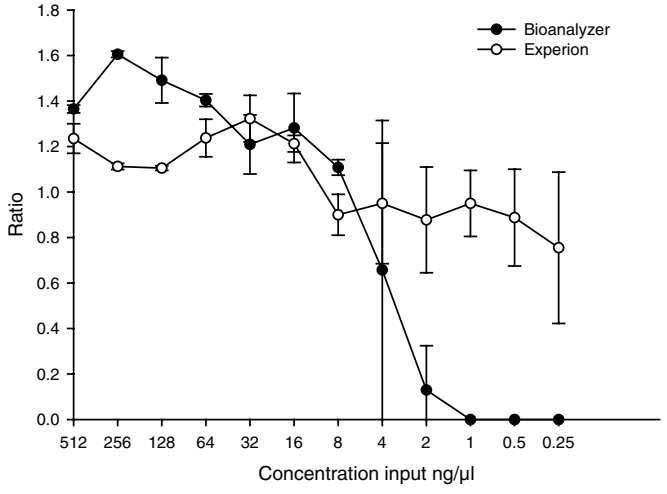



**Fig. 5.** 28S/18S ribosomal RNA Ratio versus total RNA input concentration

In regard to RNA quantitation and 28S/18S ratios, more accurate measurements are achieved by the Experion, especially at low concentration levels. The greater detection sensitivity of the Experion system allows the generation of a higher signal-to-noise ratio. In consequence the consumption of smaller amounts of RNA sample per measurement is possible.

**Validity of the RNA ladder run**

The ladder electropherogram of the Experion and the 2100 differ from each other concerning the number and sizes of the contained fragments. The Experion ladder electropherogram shows nine peaks, whereas the 2100 Bioanalyzer ladder displays only seven. Both ladders were pipetted in both separation systems; in the ladder well and in the fist sample well (figure 6). The ladder appearance and the ladder quality were evaluated in 37 runs on each platform. 62.2% of all ladder runs and 75.7% of all sample runs made with the Experion can be declared as good. In contrast, 86.5% and 88.3% of both the ladder and the sample runs provided by the 2100 Bioanalyzer are defined as successful runs. Higher validity and lower fluctuation of the ladder run is given by the 2100 Bioanalyzer system.

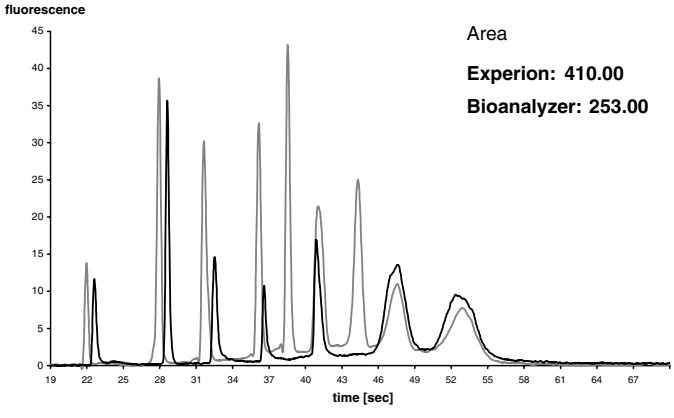



**Fig. 6.** Electropherogram of both ladders. Experion (dotted line) and 2100 Bioanalyzer (solid line)

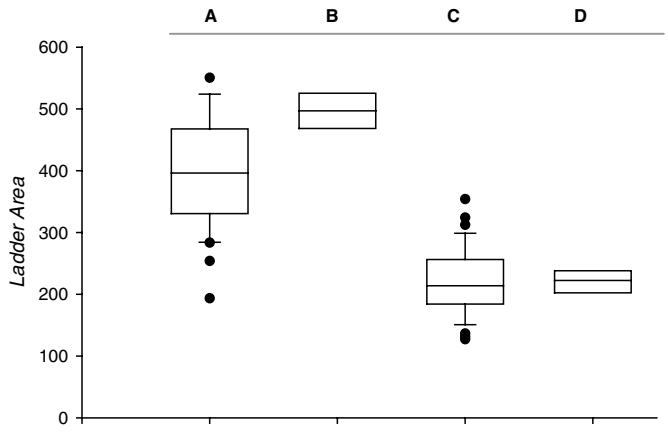



**Fig. 7.** Comparison of the ladder area on both capillary electrophoresis systems. (**A**) Experion ladder area (n = 37); (**B**) 2100 Bioanalyzer ladder performed in Experion (n = 4); (**C**) Bioanalyzer ladder area (n = 37); (**D**) Experion ladder run performed in 2100 Bioanalyzer (n = 4)

The Experion showed an average ladder area about 400.6 area units with a variance of 24.0%. The 2100 Bioanalyzer showed a variance of 24.2% and a smaller average ladder area unit about 218.8 (table 1; figure 7). Therefore the reproducibility of the ladder area for both systems is equivalent. Agilent Technologies defines a ladder area around 200 area units as normal; this corresponds to a concentration of 150 ng RNA. In contrast, Bio-Rad does not have a definition of a ladder area. Measuring the ladders vice versa on the both platforms, the Experion Ladder performed in the 2100 Bioanalyzer show a CV of ~8.5% and the Experion show a CV of ~ 8.1% for analyzing the 2100 Bioanalyzer ladder. The low statistical spread in C and D can be explained by considering that only a limited number of samples were used (figure 6).

**Comparison of the Runs**

Higher validity and lower fluctuation of the ladder run was given by the 2100 Bioanalyzer system. The Experion ladder area is roughly two-fold of the internal area units, therefore the 2100 Bioanalyzer and so the standard deviation as expected if the two systems were fully comparable. This indicates that the reproducibility of the ladder areas for the two systems is equivalent, regardless whether or not the value is stated.

## Conclusions

The Experion system showed greater sensitivity of detection and provides a better quantity assessment of RNA samples. The RNA concentration measurements are less accurate and less reproducible, as they are most similar to the UV-Spectrophometer or the NanoDrop 1000. The reproducibility of both systems is nearly identical when used for RNA quantitation. The distribution of data illustrates that the chip-to-chip variations in both accuracy and reproducibility were very comparable. One advantage of the 2100 Bioanalyzer is that the system offers the opportunity of two quality measurements; the 28S/18S ribosomal RNA ratio and the RNA integrity number (RIN), whereas the Experion only offers the ribosomal ratio. It was revealed, that the ribosomal 28S/18S ratio is inadequately

to describe RNA integrity. A general recommendation couldn't be expressed, because both systems offer same applications and good handling. The Experion system is more convenient through the automatic priming station, which might be the reason of more reproducibility and higher sensitivity in the lower RNA range.

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