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How pertinent is the RIN for validating RNA quality isolated from cell-free biological fluids?

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Introduction

Pure and high-quality RNA is essential for achieving reliable sequencing (RNA-seq) data. Therefore, before constructing RNA-seq libraries, the quality of the RNA should be evaluated (Green, 2012). Current sequencing platforms frequently suggest evaluating the RNA quality using the RNA Integrity Number (RIN).

RIN as an RNA quality indicator

The RIN is a quality control measurement that provides information about the overall quality and integrity of the RNA in a sample. It is defined not as the “relative abundance of a full-length transcript,” but rather as a computational algorithm that classifies electrophoretic RNA profiles based on multiple features, including the ratio of ribosomal RNA bands and the presence of degradation products (Imbeaud et al., 2005; Schroeder et al., 2006). The RIN value ranges within a scale of 1–10, with higher values indicating higher RNA integrity, while lower values indicate increasing levels of degradation. Generally, a RIN score equal to or greater than six is the minimum threshold considered suitable for high-quality total RNA intended for transcriptomics (Schroeder et al., 2006). The RIN is calculated based on the electrophoretic profile of total RNA obtained using an automated electrophoresis system, such as the Agilent Bioanalyzer or Agilent TapeStation, which separates on a microfluidic chip or tape RNA molecules based on their size and measures the degree of fragmentation and degradation (<https://www.agilent.com>). The resulting electropherogram is analyzed by proprietary software that evaluates multiple features, including the 28S:18S ribosomal RNA ratio, the height and area of ribosomal peaks, the presence of fast-migrating degradation fragments, and the baseline signal between peaks. As compared with ribosomal RNA (rRNA), the quantity of messenger RNA (mRNA) and other small RNA in the cell/tissue is very small, around 3–7% of the total RNA depending on the physiological/developmental stage of the cell, and unlike rRNA, the other RNA types has variable transcript length. Furthermore, the 28S and 18S ribosomal subunits are the most prevalent species among rRNA and account for about 80–90% of the total RNA in the cell (Deng et al., 2022). Therefore, the RNA integrity calculation algorithm is based primarily on the ratio of 18S to 28S rRNA (Imbeaud et al., 2005; Schroeder et al., 2006).

The limitations of RIN

The RIN calculation, however, is not an ideal method to estimate the RNA quality due to the following reasons:

1. It relies on the quality and quantity of rRNA (18S and 28S), which is always higher in cells than the total mRNA at any stage of development in any cell type (Deng et al., 2022).
2. There is no way to determine the intactness of mRNA, which is used for library preparation.
3. Also, the RIN does not contain any information on the quality of other RNA types present in the sample that, in some cases, are important for library preparation, such as non-coding RNA, microRNA (miRNA), or small interfering RNA (siRNA).
4. There is evidence in the literature that miRNAs have enhanced stability compared to rRNA (Mitchell et al., 2008; Hall et al., 2012; Ludwig et al., 2017; Jung et al., 2010) suggesting that miRNA can be of high quality, even in old samples with a low RIN.
5. FFPE (Formalin-Fixed Paraffin-Embedded) derived RNA samples which tend to be highly degraded and typically have low RIN values, can still be successfully sequenced. (Marczyk et al., 2019). Similarly, even extensively degraded forensic samples achieved high-quality output with >80% of sequences above Q30 and were mapped successfully sequenced and mapped to reference genomes (Everaert et al., 2019).

Despite the abovementioned limitations, RIN continues to be a frequently used method for assessing RNA quality in many fields of current biology research (Stephenson et al., 2020; Jaiaue et al., 2021; Kvastad et al., 2021; Wani et al., 2022; Zheng et al., 2023; Xie et al., 2025).

RIN measurements in cell-free biological fluids

In 2009, Ibberson et al., reported that in degraded total RNA samples with a low RIN, miRNA expression could not be accurately profiled. They further recommended using RNA samples with a RIN of seven or above for miRNA profiling (Ibberson et al., 2009). It appears that this train of thought is still widely accepted. Nevertheless, Ibberson standardized their experiment on solid biological samples and did not test any biological fluids in their study. To the best of our knowledge, studies which demonstrated high-RIN scores of mRNA or miRNA isolated from cell-free biological fluids have not been reported.

The RIN-based RNA quality assessment becomes futile when the sample comprises biological (cell-free) fluids containing mRNA, miRNA, small interfering RNA (siRNA), or other small RNA. Cell-free biological fluids, especially those secreted from cells, and exosomes are not expected to contain organelles or cellular rRNA; they sometimes contain small RNAs, including miRNA or siRNA, apart from mRNA. Thus, the RIN for such biological samples (plasma, serum, saliva, urine, milk, cerebrospinal fluid, or plant exudate) is always low compared to that of solid tissue and generally in the range of 2–3.

Several studies of RNA extracted from human biological fluids successfully profiled miRNA-seq libraries, without providing (or even checking?) the sample's RIN (Rahimian et al., 2023). For example, Qin et al. showed the TGIRT- (thermostable group II intron reverse transcriptase-seq) sequencing of human plasma miRNA samples. The bioanalyzer electrogram provided in their study, clearly indicates the absence of 18S and 28S peaks, suggesting a low RIN (Qin et al., 2016). Similarly, RIN was not provided by Tokuhisa et al

who studied exosomal miRNAs extracted from malignant ascites and peritoneal lavage fluid. By using Agilent 2100 Bioanalyzer, they showed that the 18S and 28S peaks were absent from these samples, indicating that the exosomal miRNA was not contaminated with intracellular RNA (Tokuhisa et al., 2015). Likewise, Channavajhala et al. who studied exosome-extracted miRNAs from urine samples, showed their RNA preparations lacked ribosomal RNAs, without providing the sample's RIN (Channavajhala et al., 2014). In all these studies the miRNA-seq libraries were successfully sequenced and profiled, despite the absence of a high RIN. It is noteworthy that, the 2023 published QIAGEN QIAseq® miRNA Library Kit Handbook states that: "It is not useful to assess the RNA integrity of total RNA derived from fluids and/or exosomes." (<https://www.qiagen.com/us>, <https://share.google/KNyk11QJ5RQell6JB>).

The case of RIN in flower stigma exudates

In line with these studies we recently reported the identification and library preparation of mature miRNA from the stigma exudate of pear flower (Ambastha et al., 2023). The pear stigma produces exudates (Figures 1A,B) that contain several metabolites and nucleic acids which are essential for sexual reproduction processes. As the exudates are secreted by the papilla cell of the stigma, it is a cell-free system devoid of cytoplasmic ribosomal or other organelle contamination (Ambastha et al., 2023) (Figures 1E–H). The exudate was utilized to isolate RNA, the concentration of which was low (Figure 1C) and the calculated RIN was lower than that generally acceptable, with a value of 1.8–2.8 (Figure 1D), essentially indicating that the RNA was degraded according to common interpretation of low RIN values. During the reviewing process of our manuscript, we obtained the following criticism by some reviewers which stated that: "The quality of RNA extracted from stigma exudate is low, as well as the RNA Integrity Number (RIN) is (too) low to be sequenced". Nevertheless, when used for library preparation and sequencing, the exudate RNA was successfully sequenced and resulted in a novel discovery of identifying miRNA in stigma exudates, which opens new research avenues in horticulture (Ambastha et al., 2023).

Alternative quantity and quality assessments for cell free RNA

A major advantage of using RIN values, when appropriate, is the ability to assess RNA quality prior to costly steps of library preparation, sequencing and analysis. In the cases we discussed where RIN is irrelevant, RNA purity and quantity can still be measured in several ways including RiboGreen assays, spectrophotometric measurements, and Bioanalyzer analysis (Williams et al., 2013; Burgos et al., 2013; Danielson et al., 2017). In addition, sample sequencing preparation can be simplified by several library preparation kits which are on the market (Tesovnik et al., 2021). Here we wish to highlight the Qubit and Quant-iT microRNA assays which use a fluorescence-based approach to accurately measure small RNA molecules, such as miRNA and siRNA (bp70-all-flr.pdf). Unlike traditional methods such as A260 spectrophotometry or standard RNA dyes, which are better suited for longer RNA fragments, these assays use a special dye that greatly increases fluorescence (over 200 times) when binding to small RNAs (17–25 nucleotides) and minimally responds to large RNAs (over 1,000 nucleotides). The system can detect RNA at concentrations

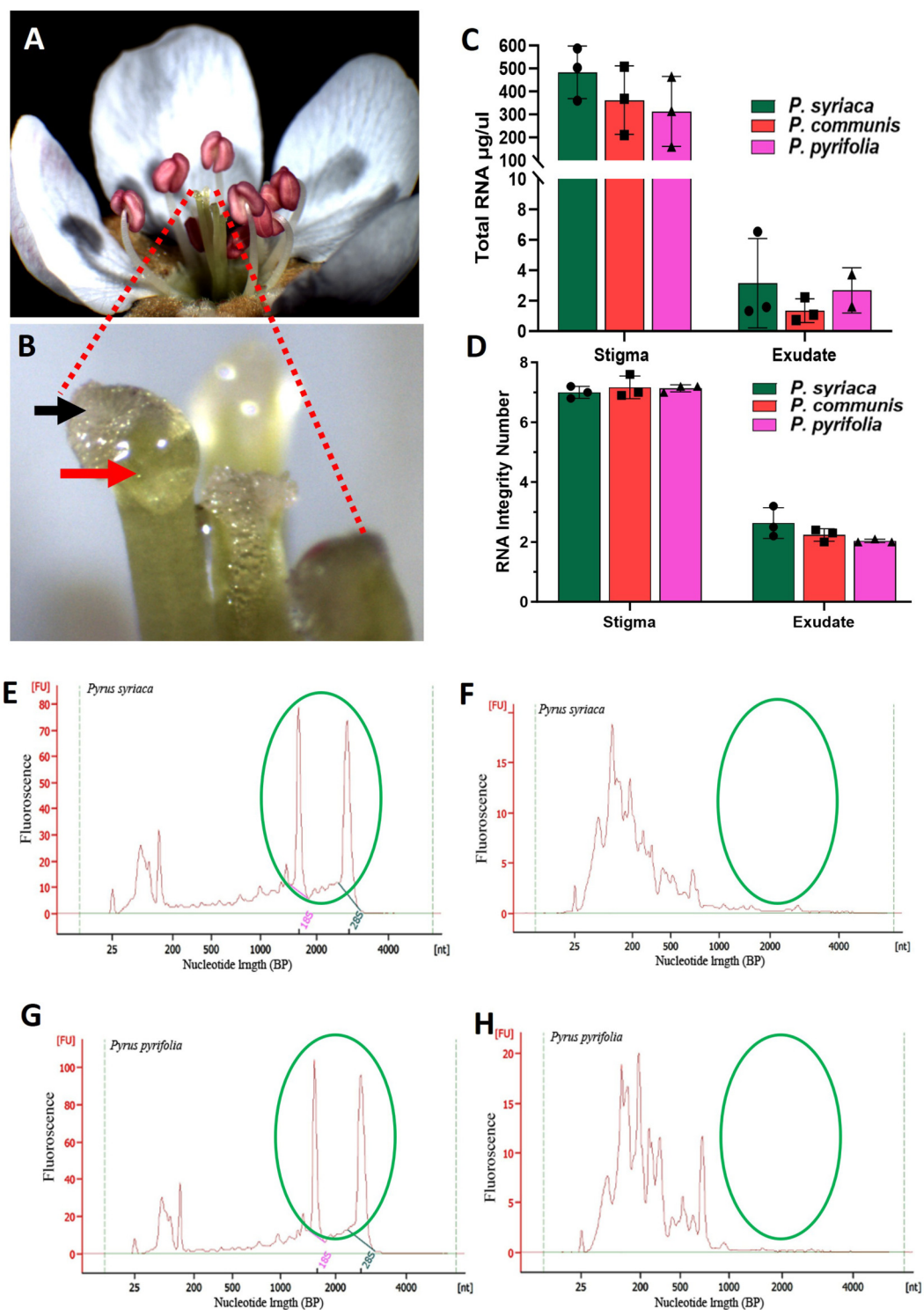


FIGURE 1

RIN assessment of RNA isolated from stigma and stigma exudate of pear flowers. (A) The pear flower (*Pyrus communis*) 12 h after anthesis (HAA). (B) Zoom-in image of the stigma lobes of the flower presented in (A). The black arrow indicates the stigma surface while the red arrow points to the exudate secretion. Both images were captured by trinocular stereoscope. (C) Total RNA concentrations of RNA sampled from stigma and stigma exudates of three pear species. (D) RIN values of the RNA samples presented in (C). Each bar in C and D represents the mean value of three independent readings $\pm$ SE. In (C,D), each stigma sample was extracted from at least 10 stigmas sampled from flowers at 12 HAA, while each exudate sample was collected in a non-destructive manner from stigmas of at least 500 flowers. (E–H) Representative electropherograms for RNA isolated from stigma and stigma exudate of *Pyrus syriaca* (E,F), respectively, and *Pyrus pyrifolia* (G,H), respectively. Note the sharp peaks of 18S and 28S RNA which are visible in the stigma samples (E,G) but completely absent in the exudate samples (F,H), marked with a green ellipse. The RNA samples were electrophoretically assessed by Agilent 2100 Bioanalyzer. The details of RNA extraction and evaluation procedures were fully described in our recent publication (Ambastha et al., 2023).

as low as 50 ng/mL and works well even in the presence of common contaminants like salts, proteins, and free nucleotides. This makes it reliable for accurately quantifying miRNA and siRNA from small or difficult samples, such as cell-free fluids and exosomes. The technique is currently considered the best method for miRNA quantification because it avoids the measurement inaccuracies of traditional RNA assays and provides precise molecule-specific results for small non-coding RNAs. (Garcia-elias et al., 2017; Gupta et al., 2020). Nevertheless, we are not aware of a way to measure RNA integrity prior to sequencing. The quality of the RNA can be measured post analysis, by checking RNA types composition, percentage of full-length small RNA molecules (often shorter than the read length), or checking uniform coverage for longer molecules by using TIN values (Transcript Integrative Number) (Wang et al., 2016).

Concluding remarks

In conclusion, the RIN possesses several limitations that apparently make it only relevant and applicable to biological tissues. Our own experience, as well as the various cases cited here, demonstrate RIN values are irrelevant for the quality estimation of cell-free small RNA, where 28S and 18S rRNA is absent. It is plausible to expand the same reasoning to other cases where rRNA is not well represented, such as mRNA sequencing from cell-free systems or specific cellular compartments, e.g., RIP-Seq experiments. Therefore, those performing such sequencing should be aware of the possibility that the RIN value obtained may not accurately reflect the RNA quality.

If the sRNA sample originates from a cell-free biofluid, the RIN loses its relevance, and becomes meaningless and misleading, indicating low-quality RNA, when in fact, the quality may be high. We have indeed shown that despite a low RIN, high-quality sequencing can thus be achieved. RNA quality assessment is certainly important, however, the desire for high RIN scores is not realistic in the case of small exRNA (plant and other organisms) and seems to our opinion to impose an obstacle to RNA research in cell free systems.

Author contributions

VA: Validation, Formal Analysis, Methodology, Investigation, Writing – review and editing, Conceptualization, Writing – original draft. YN: Conceptualization, Writing – original draft, Investigation, Validation, Formal Analysis, Writing – review and editing,

Methodology. YL: Investigation, Conceptualization, Funding acquisition, Writing – original draft, Writing – review and editing, Project administration.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declared that generative AI was not used in the creation of this manuscript.

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