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Forum

Methodological considerations for circulating long noncoding RNA quantification

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In the past decade, significant resources have been invested in long noncoding RNA (lncRNA) research. Despite the knowledge available, we are far from incorporation of lncRNA into clinical practice. Here, we emphasize the technical challenges in the field, hoping to provoke a response leading to new consensus and guidelines.

Although a potential clinical application in medical decision-making has been suggested for circulating cell-free lncRNAs [1], the majority of the novel findings have failed to be validated across different investigations. This is in part due to technical challenges and lack of standardized methods. Currently, the available literature does not provide recommendations for preanalytical and analytical variables to be considered during circulating lncRNA quantification. Moreover, highly heterogeneous strategies are often incorrectly used to compensate the methodological challenges. Here, we emphasize problems that could compromise the reproducibility of circulating lncRNA quantification, in an effort to encourage the field to define consensus and guidelines on these issues.

Reference RNAs

The use of high-throughput technologies (i.e., RNA sequencing or microarrays) has enabled the discovery of a large number of disease-specific transcriptomic targets. However, because of the high cost of these methodologies, the new markers are usually validated using RT-qPCR on a larger number of samples. RT-qPCR is the gold standard technique to quantify circulating lncRNAs, yet not much consistency across different protocols can be found regarding the choice of the endogenous/exogenous control(s). Unfortunately, we are not anywhere near global consensus on that matter. Synthetic RNA spike-in templates (synthetic RNAs that lack sequence similarities to known transcripts) may be good exogenous controls to monitor the extraction, reverse transcription, and qPCR efficiency. However, exogenous controls cannot account for variations introduced before RNA isolation. An additional problem lies in the fact that, in most cases, synthetic *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p) is used as the spike-in control. Considering that this spike-in control is a miRNA, the differences associated with RNA isolation and miRNA–lncRNA quantification should be taken into account. Different spike-in mixtures for RNA quantification are commercially available, but the number of studies implementing these controls in their protocols is limited.

When it comes to the use of endogenous controls, there is a lack of consensus on appropriate stable RNAs (those transcripts with acceptable expression levels that show low variation among experimental conditions) to normalize cell-free lncRNA levels [2]. The use of rRNAs or mRNAs as endogenous controls should be avoided because these transcripts may appear in the circulation due to cell lysis, have different biochemical characteristics from lncRNAs, and display a high expression variability. To our knowledge, one of the most suitable approaches may be to perform preliminary studies using multigene datasets obtained with RNA sequencing, microarray or RT-qPCR, and algorithms such as geNorm or NormFinder. Although this approach is recommended for every new set of experiments, it is time-consuming, is demanding in terms of input biological sample requirement, and can be costly. Endogenous controls with universally stable expression levels in a disease-specific manner remain to be determined.

Timing

Blood samples are generally obtained following standardized protocols, after 12 h of fasting early in the morning in order to avoid the influence of postprandial state (lipemia) and circadian variation. However, this procedure for sample collection cannot be strictly followed in the case of sudden patient admission. Currently, there are no systematic studies that have assessed the effects of lipemia and circadian variation of circulating lncRNAs. On the one hand, considering the lipidic nature of extracellular vesicles (that are carriers of lncRNAs in the blood), it can be expected that high levels of lipoproteins in a postprandial state may affect their isolation from blood samples [3]. On the other hand, it is not known if lipemia *per se*, and to what extent, could influence the isolation of lncRNAs. In addition, some circulating RNAs and extracellular vesicles are implicated in regulatory networks of circadian rhythm, but again, to what extent this could lead to variation in levels of specific lncRNAs remains to be elucidated [4].

Source

Whether to use serum or plasma as a biological specimen constitutes a key point in the analysis of circulating lncRNA levels in blood. Decade-long experience in the quantification of circulating miRNAs showed that serum and plasma miRNA profiles can be quite different because of the possible release of platelet-enriched miRNAs during the coagulation process [5]. In the field of lncRNAs, a large-scale deep sequencing analysis of human platelets identified more than 6000 lncRNAs [6]. Consequently,

similar to miRNAs, one should pay attention to platelets as a possible source of preanalytical variation when quantifying circulating lncRNAs. Hemolysis due to red blood cell rupture during blood collection and processing is also a potential confounding preanalytical factor that may alter the pool of circulating lncRNAs [7].

Importantly, lncRNAs are sensitive to degradation by exonucleases (RNases), and it is critical, in order to limit this degradation and ensure good-quality and reproducible results, to process and freeze serum/plasma samples within 2 h after blood draw, avoid freeze–thaw cycles, and use an RNase-free working environment. As far as RNA extraction is concerned, several well-validated approaches are commercially available, based on either column extraction or capture beads. Both approaches can be semiautomated and work fine if handled in an RNase-free environment.

Primer design

Another critical step in the quantification of lncRNAs is the design of PCR primers. lncRNAs are often present in multiple isoforms that exhibit different or even opposite functions [8]. Therefore, the choice to design primers targeting one, multiple, or all isoforms can drastically affect results and their ability to be reproduced in different laboratories [9]. Since lncRNAs are often found within protein-coding genes, on a sense or antisense strand, primers need to be carefully designed to specifically target the noncoding sequence and not the coding mRNA sequence derived from the same gene.

qPCR protocols

Because many lncRNAs are not abundant in the circulation, the addition of a preamplification step before quantification may be crucial for lncRNA analysis. Preamplification is one of the points that raises a lot of debate and controversy in

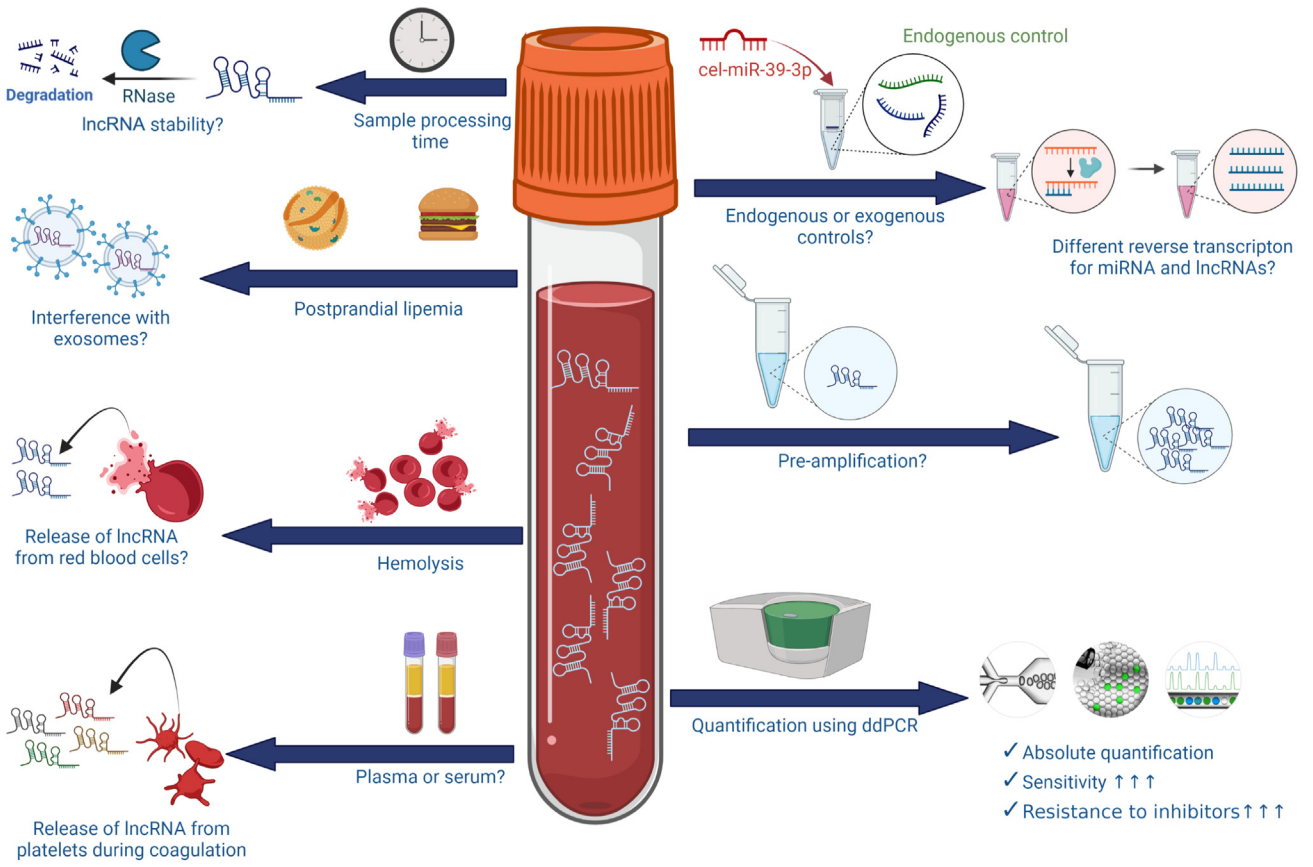

Figure 1. Considerations for circulating cell-free long noncoding RNA (lncRNA) quantification. (i) Preanalytical (from the upper left to the bottom left): sample processing time and lncRNA stability, postprandial lipemia interference with exosomes, hemolysis interference with red blood cell-specific lncRNAs, and different lncRNA profiles in serum and plasma due to coagulation. (ii) Analytical (from the upper right to the bottom right): reference RNAs (endogenous and/or exogenous) for normalization, preamplification protocols for detection of low-abundance lncRNAs, and use of more sensitive methods [such as digital droplet PCR (ddPCR)]. This figure was created with BioRender.

lncRNA quantification, with divergent results available in the state-of-the-art probably due to technical biases introduced in the preamplification-quantification workflow. In the study by Schlosser *et al*. [10], with the exception of LIPCAR, the majority of lncRNAs tested in plasma were undetectable or sporadically detectable despite preamplification. Iempridee *et al.* [7] demonstrated that target-specific preamplification causes a decrease of approximately five PCR cycles and proposed that this approach could be used to overcome the problem of low abundance. In the study by Jin *et al.* [11], preamplification also improved the raw Cq values of HULC lncRNA, but the relative expression levels were not significantly different.

In addition to the preamplification protocols, other methodologies could be used for quantification of low-abundance circulating lncRNAs. Digital droplet PCR (ddPCR) is a very sensitive technique that enables precise absolute quantification of low-abundance RNA and DNA targets [12]. So far, there are not much data on the use of ddPCR in lncRNA quantification. Considering that ddPCR is a much more sensitive tool that also enables absolute quantification of targets, and that absolute quantification is much more suitable for clinical applications, it seems that this technology could facilitate the bench-to-bedside translation of lncRNA biomarkers.

Concluding remarks

In summary, critical factors, from blood collection and processing to quantification, should be taken into account to fully address the potential clinical application of circulating lncRNAs (Figure 1). In this scenario, there is an urgent demand for best practices guidelines and standard operating procedures in order to overcome current weaknesses in terms of reproducibility. Much remains to be done, such as the definition of quality controls and calibrators, which will allow advancing the technology readiness level of lncRNAs as a novel class of biomarkers for clinical use.

These collaborative approaches should include not only laboratory specialists but also basic researchers and industry partners, thus creating a multidisciplinary environment that will catalyze the translation of circulating lncRNAs from bench to bedside.

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Declaration of interests

Y.D. holds patents related to diagnostic and therapeutic applications of RNAs. The other authors have no interests to declare.

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