



**HAL**  
open science

## Assessment of salivary microRNA by RT-qPCR: Challenges in data interpretation for clinical diagnosis

Marc van der Hofstadt, Anna Cardinal, Morgane Lepeltier, Jérémy Boulestreau, Alimata Ouedraogo, Malik Kahli, Pierre Champigneux, Laurence Molina, Franck Molina, Thi Nhu Ngoc Van

### ► To cite this version:

Marc van der Hofstadt, Anna Cardinal, Morgane Lepeltier, Jérémy Boulestreau, Alimata Ouedraogo, et al.. Assessment of salivary microRNA by RT-qPCR: Challenges in data interpretation for clinical diagnosis. PLoS ONE, 2024, 19 (12), pp.e0314733. 10.1371/journal.pone.0314733 . hal-04796770

HAL Id: hal-04796770

<https://hal.science/hal-04796770v1>

Submitted on 13 Jan 2025

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

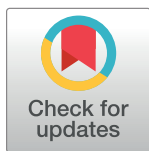
## RESEARCH ARTICLE

# Assessment of salivary microRNA by RT-qPCR: Facing challenges in data interpretation for clinical diagnosis

Marc Van Der Hofstadt<sup>1</sup>, Anna Cardinal<sup>1</sup>, Morgane Lepeltier<sup>1</sup>, Jérémy Boulestreau<sup>1</sup>, Alimata Ouedraogo<sup>1</sup>, Malik Kahli<sup>1</sup>, Pierre Champigneux<sup>1</sup>, Laurence Molina<sup>1</sup>, Franck Molina<sup>1\*</sup>, Thi Nhu Ngoc Van<sup>1,2\*</sup>

**1** Sys2Diag UMR9005 CNRS/ALCEN, Cap Gamma, Parc Euromédecine, Montpellier, France, **2** SkillCell/ALCEN, Cap Gamma, Parc Euromédecine, Montpellier, France

\* [thi-nhu-ngoc.van@sys2diag.cnrs.fr](mailto:thi-nhu-ngoc.van@sys2diag.cnrs.fr) (TNNV); [franck.molina@sys2diag.cnrs.fr](mailto:franck.molina@sys2diag.cnrs.fr) (FM)



## Abstract

Salivary microRNAs (miRNAs) have been recently revealed as the next generation of non-invasive biomarkers for the diagnostics of diverse diseases. However, their short and highly homologous sequences make their quantification by RT-qPCR technique highly heterogeneous and study dependent, thus limiting their implementation for clinical applications. In this study, we evaluated the use of a widely used commercial RT-qPCR kit for quantification of salivary miRNAs for clinical diagnostics. Saliva from ten healthy volunteers were sampled four times within a three month time course and submitted for small RNA extraction followed by RT-qPCR analysed. Six miRNAs with different sequence homologies were analysed. Sensitivity and specificity of the tested miRNA assays were corroborated using synthetic miRNAs to evaluate the reliability of all tested assays. Significant variabilities in expression profiles of six miRNAs from ten healthy participants were revealed, yet the poor specificity of the assays offered insufficient performance to associate these differences to biological context. Indeed, as the limit of quantification (LOQ) concentrations are from 2–4 logs higher than that of the limit of detection (LOD) ones, the majority of the analysis for salivary miRNAs fell outside the quantification region. Most importantly, a remarkable number of cross-talk reactions exhibiting considerable OFF target signal intensities was detected, indicating their poor specificity and limited reliability. However, the spike-in of synthetic target miRNA increased the capacity to discriminate endogenous salivary miRNA at the LOQ concentrations from those that were significantly lower. Our results demonstrate that comparative analyses for salivary miRNA expression profiles by this commercial RT-qPCR kit are most likely associated to technical limitations rather than to biological differences. While further technological breakthroughs are still required to overcome discrepancies, standardization of rigorous sample handling and experimental design according to technical parameters of each assay plays a crucial role in reducing data inconsistencies across studies.

## OPEN ACCESS

**Citation:** Van Der Hofstadt M, Cardinal A, Lepeltier M, Boulestreau J, Ouedraogo A, Kahli M, et al. (2024) Assessment of salivary microRNA by RT-qPCR: Facing challenges in data interpretation for clinical diagnosis. PLoS ONE 19(12): e0314733. <https://doi.org/10.1371/journal.pone.0314733>

**Editor:** Guido Sebastiani, University of Siena, ITALY

**Received:** July 4, 2024

**Accepted:** November 14, 2024

**Published:** December 10, 2024

**Copyright:** © 2024 Hofstadt et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the manuscript and its [Supporting Information](#) files.

**Funding:** The author(s) received no specific funding for this work.

**Competing interests:** The authors declare that there are no conflicts of interest.

## Introduction

In the last few decades, human saliva has attracted a great attention in the medical diagnostic field as a bio-fluid that provides access to various biomarkers in a non-invasive manner. Similar to peripheral blood, saliva contains a high versatility of circulating molecules that allows early diagnosis of systemic disorders [1,2]. For clinical practice, saliva offers a number of advantages over blood sampling such as a remarkable stability [1], simple sample collection, possibility for auto-sampling, and practical transportation and storage condition. In addition, saliva has a high turnover rate exhibiting fast bidirectional exchange of biomarkers, which opens access to recent biological events. However, the high turnover rate is at the cost of harbouring large biochemical and physical dynamics among samples [2] and the presence of buccal mucosa and non-human derived (*i.e.* oral bacteria) biomaterial that mask the low concentration of circulating biomarkers. Altogether, this makes the quantification of salivary biomarkers highly challenging, requiring exceedingly robust assays [3]. Nevertheless, the concentration levels of salivary biomarkers of different natures including DNA, RNA, protein and metabolite have been identified to be associated to a broad range of diseases [4–7] via numerous clinical studies, demonstrating the significant clinical interest of precisely quantifying these biomarkers.

Recently, salivary miRNAs have been considered as the next generation of non-invasive biomarkers for the diagnostics of diverse diseases [8,9]. They are small non-coding RNAs that regulate gene expression at the post-transcriptional level, playing a crucial role in every fundamental aspect of cellular function [10]. The relationships of their regulation with the disease onsets [8,9] and their presence in the extracellular circulation [11–13] make them available for various liquid biopsies enabling non-invasive medical assessment [14,15]. For instance, signatures of salivary miRNAs have been recently associated with the diagnosis of mild traumatic brain injury (mTBI) [6,16–18], cancers [12,19,20], endometriosis [21,22], neurodegenerative [23], metabolic [24] or systemic diseases [7] along with many others [25–29]. However, due to their small size (19–25 nucleotides), their high sequence similarity and their complex gene regulation process [10], the correlation between their expression levels and disease stages is often non-linear, causing it an issue of constant debate [8,15,30]. The majority of the miRNA studies have been standardized by microarray or Next Generation of Sequencing (NGS) methods followed by subsequent validation using various customized RT-qPCR approaches [31–36]. Notwithstanding the practicality, rapidity and cost effectiveness(8) of the RT-qPCR technique, its application in the quantification of miRNAs faces methodological inconsistencies in both detection and data normalization [37,38], making data interpretation controversial [39] and study dependent [18]. These unsettled topics are the main issues that restrain the use of miRNAs in current clinical practice.

Understanding technical issues of a given assay for quantification of salivary miRNA to navigate corresponding solutions would accelerate the clinical translation process for salivary miRNA biomarkers. To this aim, in this study we evaluated the use of a widely used commercial RT-qPCR kit [31] for quantification of salivary miRNAs by determining its capacity to specifically discriminate miRNAs with various homologous degrees. A panel of six miRNAs, namely hsa-Let-7a-5p, hsa-Let-7f-5p, hsa-mir-148a-3p, hsa-miR-26b-5p, hsa-miR-107 and hsa-miR-103a-3p was chosen based on both their clinical values [16,40,41] and their sequence homology. RT-qPCR assays were performed on both miRNA targets extracted from saliva of healthy volunteers and synthetic ones. Fundamental technical parameters such as sensitivity, specificity, limit of detection (LOD), limit of quantification (LOQ) and cross-reactions of all assays were characterized and included in the data interpretation process. Additionally, a synthetic version of target miRNAs spiked-in extracted sample was investigated as a potential

solution for more accurate quantification of low abundance miRNAs whose concentrations are close to or at the LOD.

## Materials and methods

### Study approval and saliva collection

Ten healthy males with ages ranging from 18 to 40 were recruited in the Sys2diag laboratory based in Montpellier, France, according to the personal protection committee (CPP) with registered number 23.00930.000169 (NCT06149351 on [www.clinicaltrials.gov](http://www.clinicaltrials.gov)). All subjects were informed, signed and consented in accordance with the CPP prior to the recruitment. Saliva collection and analyses were performed with approved protocols. Participants were asked to refrain from eating, drinking or smoking for at least 30 min prior to saliva collection. During the 3 months of the study, a total of four collections (one collection every 2–3 weeks) were performed at the same time of the day. Approximately 2 mL of unstimulated saliva was collected from each participant and stored at 4°C for a maximum of 2 hours prior small RNA extraction. The appearance of each individual saliva was visually inspected while its viscosity was estimated via its flow resistance using a combitip®.

### Salivary miRNA extraction and quantification

Total small RNA was extracted from 250 µL of whole saliva using miRNeasy Serum/Plasma Kit (Qiagen) according to the manufacture instructions (except for the elution step, where the extracted salivary small RNA was collected in 20 µL nuclease free water). In brief, 250 µL of whole saliva from each participant was homogenized in 1 mL of Qiazol solution, and was followed by the addition of  $5.6 \times 10^8$  copies of UniSP6 miRNA as a technical control for the extraction process, unless mentioned otherwise. Chloroform purification was followed by RNA precipitation by isopropanol, which was then loaded into a miRNeasy column, where only small RNA fragments (<200 bp fragments) are retained following multiple washes. All samples were handled and processed by the same manner with an equally respected delay between sampling and extraction time. Total small RNA concentration was quantified by Nanodrop One (Thermo Scientific, Wilmington USA). Small fragments of eluted RNA was confirmed by Labchip using small RNA assay (PerkinElmer), which are similar to those extracted by conventional trizol-based RNA precipitation method (S1 Fig).

### Synthetic miRNA targets

Six miRNAs, including hsa-Let7a-5p, hsa-Let7f-5p, hsa-miR-148a-3p, hsa-miR-26b-5p, hsa-miR-107 and hsa-miR-103a-3p, were selected according to their sequence similarity and clinical relevance. All the six synthetic miRNAs were purchased from Integrated DNA technologies (IDT, Europe). All sequences and annotations are available in the supplementary data section (S1 Table).

### Reverse transcription

miRCURY LNA RT Kit employing poly (A) polymerase for tailing RNA prior to an universal reverse transcription (RT) using poly T primer was purchased from Qiagen. 10 µL RT reactions were prepared in 96 well plates containing 2 µL of 5X reaction buffer, 1 µL 10X reverse transcriptase and 1 µL of synthetic or extracted small RNA at desired concentrations. The reactions were incubated in a peqSTAR 96X thermocycler (Ozyme, Montigny-le-Bretonneux France) at 42°C for 60 min, followed by a denaturation step at 85°C for 5 min and stored at -20°C until used. No template and no reverse transcriptase enzyme negative controls were

included in each run. UniSp6 miRNA was included when needed as plate calibrator. All samples and control conditions were run in duplicate.

For the analysis using 50 ng of extracted small RNA as input, all extracted samples were normalized to 50 ng/ $\mu$ L and then 1  $\mu$ L was added to the RT-qPCR reaction, except for P5 and P6 samples whose concentration were inferior to 50 ng/ $\mu$ L, and hence higher volumes were required.

### Q-PCR quantification and analysis

miRCURY LNA SYBR Green PCR Kit and target specific primers (miRCURY LNA miRNA PCR assays) were purchased from Qiagen. 10  $\mu$ L qPCR reactions were prepared in 384 multi-well plates containing 5  $\mu$ L of qPCR master mix; 1  $\mu$ L of corresponding primers (miRCURY LNA miRNA PCR Assay) and 3  $\mu$ L of 10X diluted RT product. QPCR negative controls (No-template reactions) were included for each assay. All samples and control conditions were run in duplicate. Real-time qPCR thermal cycling reactions were performed by the LightCycler 480 (Roche, Meylan France) directed by the LightCycler 480 Software (version 1.5.1.62). Thermal cycling conditions were: Pre-incubation for 2 minutes at 95°C, 40 cycles of amplification (95°C for 10s, 56°C for 60s). Ct values were analysed by Abs Quant/2nd Derivative Max of the same software. Ct values of UniSP6 was verified prior to all analysis when plate calibrator was needed. All Ct value at 35 was considered as noise.

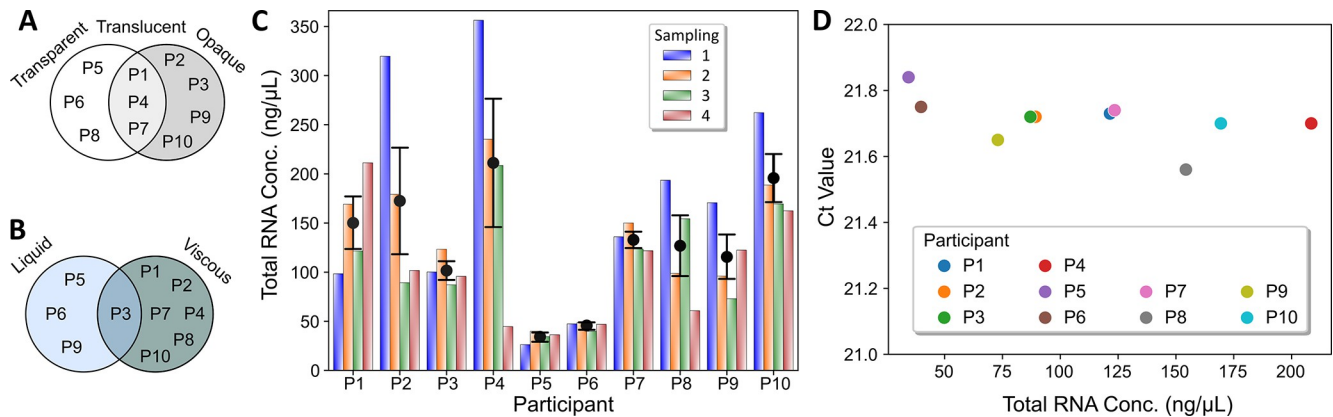
### Statistical analysis

Scipy version 1.11.2 was used on python 3.10.4 to do statistical analysis. All statistical test were done using Mann-Whitney U (scipy.stats.mannwhitneyu) with asymptotic method (*i.e.* p-value calculated by comparing to normal distribution and hence correcting for ties). All statistical calculations show the mean values  $\pm$  standard error of the mean.

## Results

### Saliva physical characteristics and their small RNA content do not affect RNA extraction efficiencies

Following each sampling, physical characteristics of individual saliva samples were visually inspected prior to small RNA extraction. Results revealed that saliva from a given participant (P) had a typical physical property harbouring a given level of cesia and viscosity, which remained unchanged throughout the 4 samplings of the study (Fig 1A and 1B). Concentrations of total extracted small RNA varied both among participants and sampling times (Fig 1C), although no significant difference among the average concentrations of the four samplings was observed (S1 Fig). We highlighted consistently low concentrations across the study for P5 and P6 samples (S2 Fig), whose saliva were both liquid and transparent. Efficiencies for every individual small RNA extraction were technically controlled by quantifying the retained amount of the UniSP6 miRNA that had been equally spiked-in preceding the extraction process. Consistent RT-qPCR results for the detection of UniSP6 (Average Ct =  $21.7 \pm 0.02$ ) using 1  $\mu$ L of extracted small RNA from all samples revealed a comparable extraction efficiency for all participants (Fig 1D), which was conserved throughout this study (S3 Fig). More precisely, upon standardizing the total RNA concentration with respect to 1  $\mu$ L condition, UniSP6 detection remains linear demonstrating that the small RNA content of a given sample does not affect its extraction efficiency (S4 Fig). Subsequently, we validated that the addition of this spiked-in miRNA had no influence on the extraction efficiency for the six target miRNAs. To this aim, we chose three samples (P2, P6 and P7) whose small RNA contents are in different



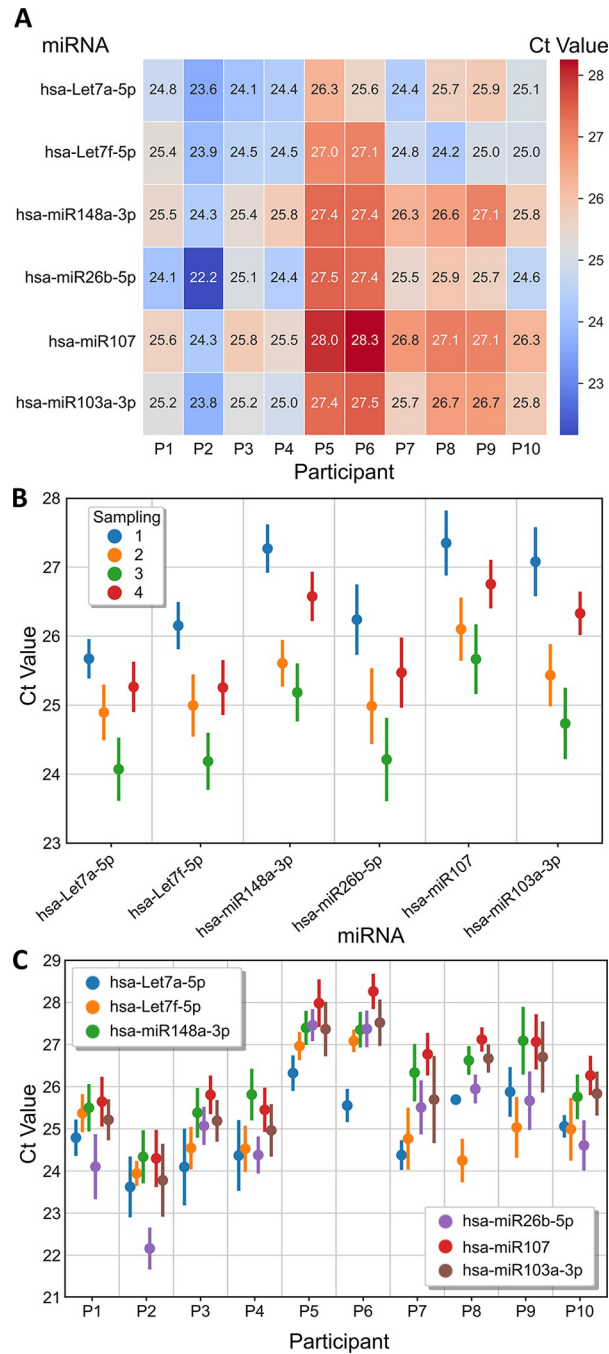
**Fig 1. Physical properties and small RNA extraction of saliva samples from 10 healthy participants.** (A) Cesia and (B) viscosity of the saliva samples prior RNA extraction. (C) Total small RNA extracted from 250  $\mu$ L of saliva for each participant and for the 4 sampling points. Black points represent the mean value and error bars show the standard error of the mean. (D) Detection of spiked artificial UniSP6 miRNA prior to RNA extraction for sampling three. 1  $\mu$ L of each extracted RNA sample was used as input. P1-10: Saliva sample from participants 1 to 10.

<https://doi.org/10.1371/journal.pone.0314733.g001>

range (Fig 1C) to perform the RNA extraction procedure in the presence or absence of the spiking UniSP6 miRNA (S5 Fig). Results of RT-qPCR analysis using 50 ng of each extracted small RNA from these samples showed that spike-in UniSP6 had no differential effect on the six assessed miRNAs as the observed Ct values increased by less than 1% (Average  $\Delta$ Ct  $0.2 \pm 0.02$ ), and (S5C Fig).

### Higher variation in salivary miRNA expression profile across participants than that of sampling points

A panel of six miRNAs was analysed on each saliva sample (10 participants and 4 temporal sampling points,  $n = 40$ ). 50 ng of extracted small RNA was used as input for universal poly (A)-tailed RT reactions followed by target specific qPCR quantification (Qiagen). Fig 2A and S2 Table show the heterogeneous expression profiles obtained from detection signals of the six target miRNAs with averaged Ct values for the four samplings ranging from 22 to 29, which are within the acceptable detection ranges considered by the community [19,42]. However, the six miRNA targets shared a similar detection pattern among the four sampling points (Fig 2B). We observed a reduction in Ct value from the sampling 1 to sampling 3, followed by a restoration in the sampling 4 whose Ct values fell between the first two samplings. Even if we had previously observed no significant difference between the average of the extracted total RNA concentrations across temporal sampling (S1 Fig), Ct values on 50 ng samples revealed the contrary, showing a significant difference between sampling 1 and 3 for all miRNAs, and between sampling 3 and 4 for half of the miRNA assessed, namely hsa-Let-7a-5p, hsa-miR148a-3p and hsa-miR103a-3p (S6A Fig). Statistical analysis for individual miRNA targets revealed the apparition of two major groups, a lower Ct value group containing hsa-Let-7a-5p and hsa-Let-7f-5p, and a greater Ct value group containing hsa-miR148a-3p and hsa-miR107 (Figs 2C and S6B). In between both groups, we found hsa-miR26b-5p and hsa-miR103-3p, which shared proximity with the lowest and the greatest Ct value group, respectively. However, the evaluation of the Ct values with respect to individual participants showed that the average miRNA signals (Fig 2B) partially masked the ones of individual participants (Fig 2C). Further analysis on these miRNA expression profiles revealed significant differences among participants, which can be clustered into three major groups: A) high Ct values (P5 and P6), B) moderate Ct values (P8 and P9) and C) low Ct values (P1, P2, P3 and P4), with participant P7 and



**Fig 2. RT-qPCR shows high variability of salivary miRNA expression profiles within 10 healthy participants.** (A) Average Ct values of the four sampling points through which the six miRNAs were assessed on 10 participants. Expression profiles of the six analysed miRNAs across (B) time and (C) different participants. All analysis was performed with 50 ng of total extracted small RNA. All values represent the mean value and the error bars show the standard error of the mean.

<https://doi.org/10.1371/journal.pone.0314733.g002>

participant P10 between the later groups, being adjacent to group B&C and group C, respectively (S7A Fig). We noted that Ct values of these groups were in close relation to the dilution factor required to achieve the 50 ng of extracted small RNA for RT-qPCR experiments

(S3 Table). More precisely, the first group presented small dilution factors (7x and 9x), the second group intermediate (25x and 23x, followed by P7 with 26.5x) and the third group highest (ranging from 30x to 42x, with the exception of the P3). Altogether, we observed that participants had higher variability (mean delta variability of 0.19) compared to sampling points for all the miRNA assays (S8 Fig).

To fully evaluate the effect of the dilution factors on miRNA detection signals, we next performed dose dependent response experiments on three samples (P2, P6, P7). The six miRNA assays were performed using different extracted small RNA input concentrations for RT-qPCR, ranging from 1 ng up to 300 ng. Results demonstrated linear behaviour for all the six miRNAs for three participants (S9 Fig). However, while participants P2 and P7 showed high correlations coefficients ( $R^2 > 0.99$ , except for hsa-Let-7f-5p in P7), participant P6 had lower coefficients, ranging from 0.94 to 0.99. Particularly, at the highest concentration (300ng), Ct values were lower than predicted by the linear fit (S9B Fig). Similarly, although the six tested qPCR assays had comparable efficiencies, overall higher efficiencies were observed for participants P2 and P7 compared to participant P6 (S10 Fig). Nevertheless, when averaging the three participants, we observed that the miRNA expression profile was conserved throughout the tested concentration range (from 1 ng up to 300 ng of extracted small RNA) (S9D Fig). Indeed, the detection of both UniSP6 (S4 Fig) and the six miRNA targets (S11 Fig) detected linearly at different working dilutions.

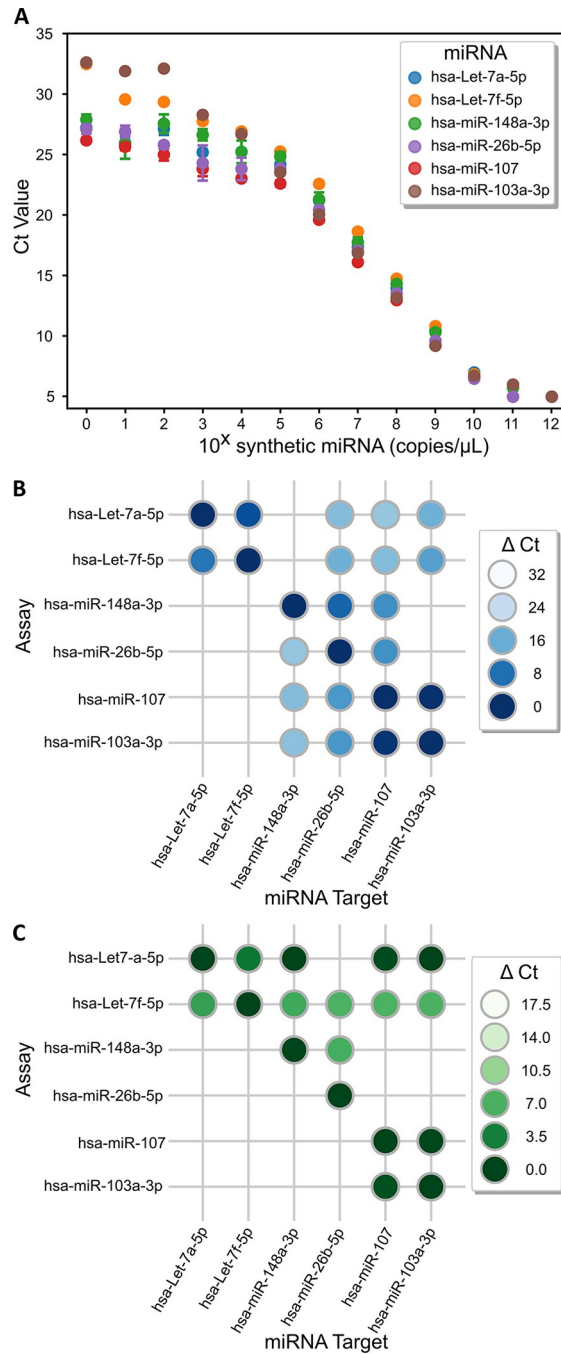
### High cross-reactivity limits the reliability of the miRNA assays

To determine to what extent the variability observed in endogenous salivary miRNA quantification was associated to biological factors, we investigated the reliability of the assessed assays. To this aim, we investigated the sensitivity and specificity of the six miRNA assays using their corresponding synthetic targets. The limit of detection (LOD) for each miRNA assay was determined by signals obtained at the lowest target concentration in a serial dilution of concentration ranging from 1 to  $10^{12}$  copies/ $\mu$ L (Fig 3A). Results demonstrated very high sensitivities, allowing to detect down to at least 1 copies/ $\mu$ L of miRNA target from their negative controls (No detection or Ct Value = 35). However, we noted that under a given concentration, the detection signals was no longer linear, and hence we defined it as the limit of quantification (LOQ). The LOQ concentrations for hsa-Let-7a-5p, hsa-miR-148a-3p, hsa-miR26b-5p and hsa-miR-107 assays were higher ( $10^5$  copies/ $\mu$ L) compared to that of hsa-Let-7f-5p and hsa-miR-103a-3p assays, which decreased down to  $10^4$  and  $10^2$  copies/ $\mu$ L, respectively. Calculation of the RT-qPCR efficiencies between the LOQ and the Ct saturation point (*i.e.* Ct value = 5) revealed high efficiencies for all miRNA assays, ranging from 98.5 and 112.5% (S12 and S13 Figs), which fall within reasonable limits for PCR exponential amplification.

Due to the short size, and hence high sequence similarity [15], miRNA detection is prone to crosstalk. To uncover the potential crosstalk among assays and targets used in this study, we compared the detection signal of each assay reporting on its corresponding miRNA target (ON target) to that of the detection on the other targets (OFF target) of the panel.  $\Delta$ Ct values were calculated between the Ct value of the OFF target and the ON target as indicator of their cross-reactivity (crosstalk).

Firstly, we assessed the crosstalk of the six miRNA assays at a single concentration of  $10^9$  copies/ $\mu$ L, which corresponds to the middle of the quantification zone (Figs 3B & S14A). Out of the 30 possible crosstalk combinations, only 12 of them showed no crosstalk (No detection or Ct Value = 35) while the rest (60%) presented significant crosstalk. The most striking crosstalk was observed between hsa-miR-107 and hsa-miR-103a-3p assays with respective  $\Delta$ Ct of 0.21 and 0.84, making their discrimination impossible. In the same line, it is very unlikely to

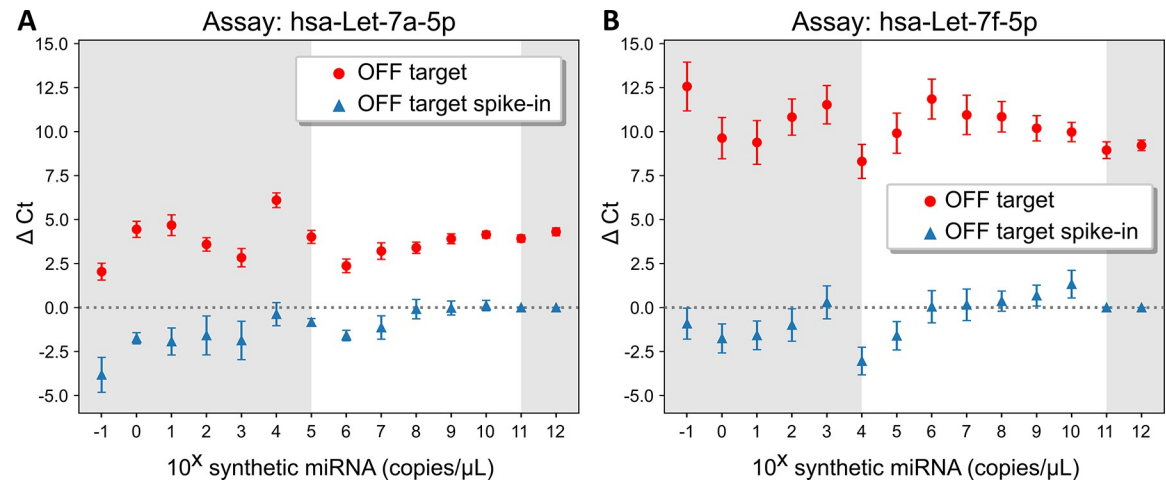




**Fig 3. Sensitivity and specificity limitations of the six miRNA assays.** (A) Serial dilution from 1 to 10<sup>12</sup> copies/μL with synthetic miRNA target for each miRNA assay to determine their limit of detection (LOD) and their limit of quantification (LOQ). Values represent the mean value and the error bars depict the standard error of the mean. Cross reactions between miRNA assays and synthetic targets at (B) 10<sup>9</sup> copies/μL and (C) 10<sup>5</sup> copies/μL. ΔCt values are calculate from S14 Fig. Empty spaces represent no crosstalk (no detection or Ct Value = 35). Data obtained from a duplicate experiment. ΔCt values = Ct<sub>OFF target</sub> - Ct<sub>ON target</sub>.

<https://doi.org/10.1371/journal.pone.0314733.g003>

discriminate hsa-Let7a-5p from hsa-Let7f-5p as these assays also presented high crosstalk, exhibiting ΔCt values of 3.9 and 8.7, respectively. The rest of crosstalk combinations presented variable ΔCt, ranging from 6.5 up to 25.4. We next assessed crosstalk contribution at 10<sup>5</sup>



**Fig 4. Conserved crosstalk for hsa-Let-7a-5p and hsa-Let-7f-5p within the quantification region.**  $\Delta$ Ct values obtained when comparing a serial dilution from  $10^{-1}$  to  $10^{12}$  copies/ $\mu$ L of the corresponding synthetic miRNA (ON target) with its cross reaction (OFF target, red circles) for (A) hsa-Let-7a-5p assay and (B) hsa-Let-7f-5p assay. The  $\Delta$ Ct by the addition of  $10^7$  copies/ $\mu$ L of OFF target on the serial dilution of ON target was also calculated (blue triangles). Red circles =  $Ct^{\text{OFF target}} - Ct^{\text{ON target}}$ , blue triangles =  $Ct^{\text{ON target + OFF target at } 10^7 \text{ copies}/\mu\text{L}} - Ct^{\text{ON target}}$ . All values represent the mean value and the error bars show the standard error of the mean (two independent experiments). Grey region delimits the quantification region.

<https://doi.org/10.1371/journal.pone.0314733.g004>

copies/ $\mu$ L, which corresponds to the LOQ concentration for four out of the six miRNA assays. At this concentration, although the number of crosstalk reduced down to 40%, most of the retained OFF targets drastically increased their significance (the average  $\Delta$ Ct decreased from 13.14 down to 3.6) (Figs 3C and S14B). As for the two pairs: hsa-miR-107 vs hsa-miR-103a-3p, and hsa-Let7a-5p vs hsa-Let7f-5p, their maximal crosstalk at  $10^9$  copies/ $\mu$ L remains unchanged when concentration was lowered to  $10^5$  copies/ $\mu$ L.

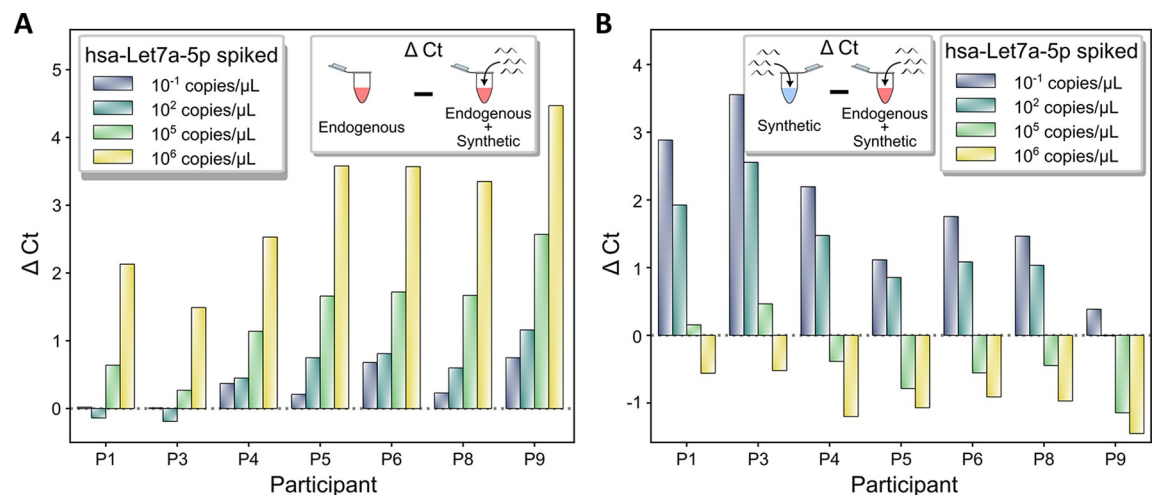
Secondly, we assessed the crosstalk between the hsa-Let7a-5p and hsa-Let7f-5p assays throughout the whole dilution range (from 0.1 to  $10^{12}$  copies/ $\mu$ L). As shown in Fig 4, throughout the whole dilution range, crosstalk between these two assays remained generally stable with an average  $\Delta$ Ct value of  $3.78 \pm 0.27$  for hsa-Let7a-5p (Fig 4A, red circle) and  $10.29 \pm 0.32$  for hsa-Let7f-5p (Fig 4B, red circle). However, we noticed erratic responses associated with higher error values when the concentration of the OFF target was lower or at the transition to the LOQ. Re-calculation of the average  $\Delta$ Ct within the LOQ zone didn't considerably change, being  $3.93 \pm 0.34$  for hsa-Let7a-5p and  $10.02 \pm 0.36$  and for hsa-Let7f-5p.

Lastly, we evaluated the influence of the OFF target at a LOQ concentration ( $10^7$  copies/ $\mu$ L) on the LOD of each assay. To do so, we compared the detection signals of the ON target throughout the whole serial dilution in the absence and presence (spike-in) of  $10^7$  copies/ $\mu$ L of the OFF target (Fig 4, blue triangle). Obtained results revealed that, for both hsa-Let-7a-5p and hsa-Let-7f-5p assays, the detection of the ON target was not affected by the presence of  $10^7$  copies/ $\mu$ L of the OFF target. However, for the hsa-Let7a-5p assay, the greatest crosstalk influence of the OFF target was observed at  $10^6$  copies/ $\mu$ L of the ON target (1 log difference), and a partial crosstalk at its adjacent concentrations (*i.e.*  $10^5$  and  $10^7$  copies/ $\mu$ L). Similarly, for hsa-Let7f-5p assay, the greatest crosstalk contribution was observed at  $10^4$  copies/ $\mu$ L of the ON target (3 log difference), showing a greater resilience compared to the hsa-Let7a-5p assay. These results are in agreement with the average  $\Delta$ Ct value of 3.9 (~ 1 log) and 10.0 (~ 3 logs) for the OFF target of the hsa-Let7a-5p and hsa-Let7f-5p assays, respectively.

## Only a minority of the miRNA detections falls within the quantification regime

Based on the quantification zone (LOQ) identified for each assay (S12 Fig), we reconsidered the term “good range of detection” stated previously in Fig 2 for the six miRNA assays. Indeed, upon taking into consideration of the LOQ (S12 Fig), we observed that out of the 240 quantified samples (10 participants x 4 samplings x 6 miRNA assays), only 29% of them were quantified in their respective LOQ zones. Consequently, up to 42% of these samples, which included all analysis for hsa-miR-107 assay, were obtained outside the quantification zone, and the remaining samples were analysed at the LOQ (particularly for hsa-Let7a-5p and hsa-miR-148a-3p). In particular, of the 29% obtained Ct values obtained within the linear quantification zone, all 40 analysis of the hsa-miR103 assay was included (as a result of its lower LOQ). These results indicated that endogenous salivary hsa-miR103-3p was present at concentrations between  $3.8 \times 10^3$  and  $5.4 \times 10^4$  copies/ $\mu\text{L}$ , and that only participant P6 showed partial significant differences with respect to the other participants (S7B Fig).

Given the high Ct values obtained for endogenous salivary hsa-Let7a-5p analysis, we decided to spike-in the synthetic hsa-Let7a-5p to surpass the LOQ concentration in order to acquire detection signal in the LOQ zone. Four different concentrations including two that were significantly under the LOQ ( $10^{-1}$  copies/ $\mu\text{L}$  and  $10^2$  copies/ $\mu\text{L}$ ), one at the LOQ ( $10^5$  copies/ $\mu\text{L}$ ) and the last one at 10-fold higher than the LOQ ( $10^6$  copies/ $\mu\text{L}$ ) were respectively spiked in the 50 ng endogenous samples. We first compared signals of the endogenous spiked-in samples with that of their respective endogenous samples alone (Fig 5A). Obtained results show a “so-called” dose dependent response in all analysed samples, since the higher the concentration of spiked-in synthetic miRNA, the stronger the detection signal (lower Ct value hence higher  $\Delta\text{Ct}$ ) (Figs 5A & S15). However, not all samples responded similarly to the different concentrations of spiked-in synthetic miRNA, where some presented only partial dose dependency. In particular, samples P1 and P3 had no signal alteration for spike-in concentrations below  $10^5$  copies/ $\mu\text{L}$ , while at higher spiked-in concentrations smaller alterations were observed (Average  $\Delta\text{Ct} = 1.81 \pm 0.26$ ) compared to other samples (average  $\Delta\text{Ct} = 3.5 \pm 0.24$ ).



**Fig 5. The spiking of synthetic hsa-Let-7a-5p miRNA allows semi-quantification at LOQ concentrations.** (A)  $\Delta\text{Ct}$  values of spike-in synthetic hsa-Let-7a-5p miRNA in 50 ng RNA extract with respect to the RNA extract alone in a range of spike-in concentrations.  $\Delta\text{Ct}$  were calculated as  $\text{Ct}_{\text{Endogenous}} - \text{Ct}_{\text{Endogenous} + \text{synthetic}}$ . (B)  $\Delta\text{Ct}$  values of spike-in synthetic hsa-Let-7a-5p miRNA in 50 ng RNA extract with respect to the respective synthetic miRNA sample alone.  $\Delta\text{Ct}$  were calculated as  $\text{Ct}_{\text{Synthetic}} - \text{Ct}_{\text{Endogenous} + \text{synthetic}}$ . Data determined from S15 Fig.

<https://doi.org/10.1371/journal.pone.0314733.g005>

However, when we compared these signals with that of their respective synthetic samples alone (Fig 5B), an opposite scenario was observed. Overall, the presence of synthetic miRNA at lower concentrations induced more alterations in signal detection in all samples whilst very high concentration negatively affected the detection of endogenous miRNA (negative  $\Delta Ct$ ). On the contrary, synthetic spiked-in miRNA at  $10^6$  copies/ $\mu L$  concentration decreased significantly the detection signal in all samples. In particular, P1 and P3 samples appeared to be those whose detection signals were the most altered displaying the most increase at the lowest concentration (average  $\Delta Ct = 3.22 \pm 0.178$ , compared to  $1.63 \pm 0.38$ ) and the least decrease (average  $\Delta Ct = -0.54 \pm 0.027$  compared to  $-1.04 \pm 0.156$ ) at the highest concentration. Moreover, the negative effect of high concentration of spiked-in synthetic miRNA was observed only from  $10^6$  copies/ $\mu L$  for these two samples, while for the remaining participants, this effect was observed at  $10^5$  copies/ $\mu L$ , allowing to discern the samples that are close to the LOQ from those that are significantly lower (semi-quantification).

## Discussion

Salivary miRNA biomarkers have become recently relevant to the medical diagnostic community due to their easiness in sample collection and their expression signatures in diverse diseases [4,6,7]. However, the lack of standardized approaches in experimental design, method of analysis and data interpretation coupled with inter-individual variability has led to the generation of considerable study-dependent and controversial data [37,39]. We evaluated the capacity to detect salivary miRNAs of a commercially available kit, which is widely used in both research and clinical studies [16,19,40]. We observed significant variability between miRNA targets and participants, although these variations cannot be fully attributed to biological fluctuations, reinforcing the need for an accurate data interpretation in clinical studies.

Our data shows that although saliva obtained from different participants vary in physical properties and small RNA concentration, this heterogeneity did not affect the efficiency of the small RNA extraction process using the chosen kit, which is by far the most sensitive [43,44] and widely used for salivary samples [16,17,21,22,40]. Interestingly, we did observe that transparent and liquid samples presented smaller variations in small RNA content throughout the four samplings and significantly lower small RNA concentrations (Figs 1C and S2). However, this low concentration of extracted small RNA did influence the downstream RT-qPCR analysis of the sample. Indeed, serial dilutions of total small RNA extracted from participant P6 also presented slightly lower correlations coefficients (S9 Fig) and partially lower efficiencies (S10 Fig) for RT-qPCR. Since higher deviations are observed at high concentrations of small RNA (300 ng), we hypothesize that the use of higher volumes (~4-fold greater compared to participant P2) would carry on more chemical residues from the purification process, which may affect the efficiency of the RT-qPCR. For this reason, and due to the conservation of the miRNA detection profile at different small RNA concentrations (S9D Fig), we propose 50 ng as input concentration for RT-qPCR as a good compromise when using human saliva samples.

Relying on already established potential miRNA biomarkers for the diagnostic of brain concussion [6,16–18], we quantified a representative panel of six miRNA (hsa-Let-7a-5p, hsa-Let-7f-5p, hsa-mir-148a-3p, hsa-miR-26b-5p, hsa-miR-103a-3p and hsa-miR-107) in saliva from ten participants at four sampling points. Interestingly, we firstly noted that although no significant difference among the average concentrations of the extracted small RNA was observed throughout the four samplings of the study (S1 Fig), upon standardization to 50 ng, a significant fluctuation of miRNA detection signal across time was obtained (Fig 2B). This could be accounted to the modification in the presence of other small RNAs (including those that are not analysed in this study), indicating that a direct relation between total extracted small RNA

concentration and miRNA concentration cannot be done. Secondly, we also observed that the temporal tendency is considerably marked for the six miRNAs assessed. We initially hypothesized that this variation may have been attributed to an environmental effect that had homogeneously affected all participants throughout the three months of the study. However, given the technical limitations described in the subsequent sections, there was not sufficient robustness to validate as the unique hypothesis.

Statistical analysis of the six miRNA targets significantly clustered them into two major groups: a low Ct value group (hsa-Let-7a-5p and hsa-Let-7f-5p) and high Ct value one (hsa-miR148a-3p, and hsa-miR107). Yet, as expected, this average miRNA expression profile partially masked the one of individual participants (Fig 2C), demonstrating the need for individualized data analysis and the risk in data clustering, especially when defining a molecular profile in clinical diagnostics. Similarly, we observed statistically significant differences among participants that have been related to their dilution factor (S3 Table), and hence to their extracted small RNA concentrations. In particular, that the two participants P5 and P6 stand out as an individual group (S7 Fig) with high Ct values. Since miRNA expression profiles of P5 and P6 were very similar to the rest of the participants (Fig 3C), there was no clear difference between the high efficiency miRNA assays and the lower efficiency ones analysing sample P6 (S10 Fig) especially at high input concentrations (300ng), where assay efficiencies are mostly affected. Therefore, we do not discard the higher Ct values of P5 and P6 due to technical limitations when using 50 ng as input. Even if miRNA expression profiles were independent from the input concentrations of extracted small RNA (S9D Fig), samples with low small RNA concentrations face a greater technical risk of being outliers and therefore should be critically taken into consideration for data handling. Given the high heterogeneity in extracted small RNA concentrations (up to 13-fold difference) and the RT-qPCR impairments due to either excessive or insufficient small RNA concentration, we discourage the use of a fixed volume of small RNA for RT-qPCR as a normalizing method [16,40].

Given the particularly small size and the possibly low abundance of the circulating miRNA targets, the fundamental parameters of the employed RT-qPCR assays should be carefully investigated prior to drawing conclusions. Our results revealed that the LOQ concentrations for the six miRNA assays were significantly higher compared to that of their LOD ones (S4 Table). Consequently, comparison of LOQ values to Ct values obtained from saliva samples revealed that only 40% of the obtained results were within the quantitative region. In particular, we could only infer quantitative information from hsa-miR-103a-3p assay, which demonstrated 1.4-fold difference between highest and lowest extracted small RNA concentration. These results highlight the technical limitations of the assays, which subsequently reduce the quantitative reliability of the obtained Ct values due to the insufficient sensitivity. Although small RNA concentration used could have been increased up to 200 ng (4-folds compared to 50 ng) to decrease Ct values and hypothetically increase up to 70% the number of results within the quantitative region, 4 out of the 6 miRNA assays would still not have all the values within the quantitative region, in addition to the cost of reducing qPCR efficiency as RNA input concentration increases.

In addition to sensitivity issues, RT-qPCR assays are commonly known to suffer from poor specificity for short targets such as miRNA(43). Our results clearly demonstrated this phenomenon, where the cross-assessment of the six miRNA assays revealed considerable cross-detection, ranging from 60% at  $10^9$  copies/ $\mu$ L (a concentration in the middle of the quantification zone, Fig 3B) down to 40% at  $10^5$  copies/ $\mu$ L (the LOQ concentration for 4 out of 6 assays, Fig 3C). However, we observed that this reduction in cross-detection was at the cost of significantly increasing the significance of the remaining ones (*i.e.* worse specificity). In particular, and as expected, we observed that miRNA assays for targets whose sequences differed by only

one nucleotide (*i.e.* hsa-Let-7a-5p from hsa-Let-7f-5p and hsa-miR107 from hsa-miR-103a-3p) were unable of discriminating one from each other (Fig 3C). In particular, we observed that for the pair of hsa-Let-7a-5p and hsa-Let-7f-5p, crosstalk remained relatively constant throughout the serial dilution of the OFF target from 0.1 to  $10^{12}$  copies/ $\mu\text{L}$  (Fig 4). Interestingly, we also noticed that the error was increasing as OFF target was decreasing, until the LOQ where the experimental errors stabilized. We attribute this behaviour to the stochastic (*i.e.* non-specific) nature of the enzymes [45] and the priming system of the assay, where at concentrations sufficiently low, the stochastic behaviour predominates over the deterministic behaviour, decreasing the robustness and linearity of amplification techniques [46,47]. Nevertheless, this high cross-detection, when carefully assessed, could beneficially be used for data interpretation. For example, although hsa-miR107 can only be quantified down to  $10^5$  copies/ $\mu\text{L}$  with the hsa-miR107 assay, due to the high crosstalk of hsa-miR107 on hsa-miR-103a-3p assay, we can use the hsa-miR-103a-3p assay to reject the presence of hsa-miR107 at lower concentrations (as low as  $3.8 \times 10^3$ , the LOQ of hsa-miR-103a-3p). Note that this statement allows us to decrease the capacity of rejecting the presence of hsa-miR107 by at least one order of magnitude only if hsa-miR-103a-3p is also absent.

Synthetic spike-in miRNAs have been widely used for technical control and signal normalization [37–39] in which cross species (*e.g.* cel 39 in human sample) or artificial (*e.g.* UniSP6) ones are generally employed. In this study, to favour the ON target amplification at the LOQ without introducing more complexity to the matrix, hence increasing signal to noise ratio, we propose to spike-in the synthetic version of target miRNA. In particular, by spiking synthetic hsa-Let7a-5p in the extracted small RNA samples we intend to acquire semi-quantitative information, which would allow us to discriminate samples that were on the LOQ from those that were significantly lower. The rationality behind this statement is that the closer (or higher) the endogenous miRNA concentration is compared to the added synthetic miRNA, the lower the impact the added synthetic miRNA will have on the Ct value (*i.e.* lower  $\Delta\text{Ct}$ ). For example, the addition of  $10^6$  synthetic copies/ $\mu\text{L}$  to a sample with endogenous miRNA at  $10^4$  copies/ $\mu\text{L}$  (ratio 100:1) will largely affect its Ct value. However, as the endogenous miRNA concentration increases to 10:1 and 1:1 ratios (endogenous miRNA at  $10^5$  copies/ $\mu\text{L}$  and  $10^6$  copies/ $\mu\text{L}$ , respectively), the lower the variation in Ct value would be observed, since the total concentration of miRNA (endogenous plus synthetic) does not increase as significantly. With this rationality, we hypothesize that participant P1 and P3 have a concentration  $\sim 10^5$  copies/ $\mu\text{L}$  (although there is 0.68  $\Delta\text{Ct}$  between them) while the other participants (P4, P5, P6, P8, P9) have concentrations  $< 10^5$  copies/ $\mu\text{L}$ , since the addition of  $10^6$  synthetic copies/ $\mu\text{L}$  had low effect on the CT values of participant P1 and P3 but a significant effect for the other participants (Fig 5B). We do not venture in predicting the concentration of the latter group (or their order) since the addition of  $10^5$  synthetic copies/ $\mu\text{L}$  is at the LOQ and hence is highly prone to error.

Highly homogenous miRNAs and those of the same family may generally share the same respective mRNA targets and, up to date, they seem to have the same biological functions. However, as to their clinical relevance, when only one member of the family is identified in a panel of biomarker, cross-detection between this particular miRNA and other member of the family would have a critical impact in data interpretation. For instance, in a big clinical study on sport-related concussion, Di Pietro and co-workers [16] showed that salivary hsa-Let7f-5p stood out as a biomarker with the highest area under the curve (AUC = 86%) allows distinguishing concussed and non-concussed patients, while the hsa-Let7a-5p was also pointed out as a marker in a panel consisting of other small RNAs. Moreover, even if the extent of crosstalk between two miRNA assays is related to their sequence similarity, our data showed the presence of crosstalk among all studied miRNAs even from those whose sequences are different (S16 Fig).

Taken together, even if only one extraction and one RT-qPCR kit were investigated in this study, our results demonstrate the importance to address the reliability of any chosen RT-qPCR kit when quantifying salivary miRNAs, since data can be highly misled by technical limitations. Including the technical constraints, such as input concentration ranges, LOD, LOQ, PCR efficiency and cross-detection (and others within the MIQE guideline [48]), are necessary to support data interpretation, allowing to distinguish the biological relevance of the RT-qPCR results from variations associated with technical and methodological limitations. Other commercially available miRNA quantification chemistries such as TaqMan, Stem-loop RT-qPCR or digital PCR should be tested as they may provide better performances.

## Conclusions

In this work we have shown that a commercially available RT-qPCR kit shows significant variabilities in expression profiles of the six salivary miRNA targets among the ten healthy participants. However, we demonstrate that the majority of these differences are associated to technical limitations rather than the biological contexts. Our data demonstrated that assessing the technical limitations of the kit coupled with a rigorous experimental design is crucial for interpreting biological relevance using RT-qPCR data. Regarding the high potential application of salivary miRNA in medical diagnosis, further technological breakthroughs for reliable tests are still required to overcome data inconsistencies and study dependent results.

## Supporting information

**S1 Fig. Average total extracted small RNA concentration does not vary significantly among sampling points.** (A) Distribution of the total extracted small RNA concentrations among 10 participants throughout four sampling points. Black points represent the mean value and error bars show the standard error of the mean. (B) Mann-Whitney U results of statistical tests performed on panel A. (C) Gel and (D) electropherogram Labchip data show similar small RNA profiles by two different extraction methods, RNA precipitation or filter column (used in this study).

(TIF)

**S2 Fig. Mann-Whitney U results for the total extracted small RNA concentration highlighting the presence of a distinct group containing participant P5 and P6.**

(TIF)

**S3 Fig. Homogenous RNA extraction efficiencies throughout the study.** (A) RT-qPCR quantification values of spike-in UniSP6 miRNA using 50 ng of the total extracted salivary small RNAs for the different sampling points. Points represent the mean value and error bars depict the standard error of the mean. (B) Mann-Whitney U results performed on panel A.

(TIF)

**S4 Fig. Ct values of the spike-in UniSP6 miRNA quantified by RT-qPCR are associated with the dilution factors of the extracted small RNA samples.** All samples were analysed in two conditions: Using 1  $\mu$ L (circle) or 50 ng (cross) of the total extracted small RNAs as input. Since the same concentration of UniSp6 miRNA is present at 1  $\mu$ L for all participants, all 50 ng values have been normalized with respect to the RNA concentration at 1  $\mu$ L (dilution factor), and all Ct values have been shifted with respect to the 1  $\mu$ L value (causing the overlapping of all 1  $\mu$ L values).  $\Delta$ Ct values =  $Ct^{50ng} - Ct^{1\mu L}$ .

(TIF)

**S5 Fig. Spike-in of artificial UniSP6 miRNA does not significantly interfere with neither six miRNA assays nor ten participants.** Small RNAs were extracted from three saliva samples (P2, P6 and P7) in the presence and absence of spiked-in artificial UniSP6 miRNA. (A) RT-qPCR quantification for the six miRNAs using 50 ng of small RNAs. Mann-Whitney U results for panel A of  $\Delta Ct$  between with and without spiking with respect to (B) individual participants and (C) miRNA assay.

(TIF)

**S6 Fig. Average Ct values analysing 50 ng small RNA input were dependent on the sampling point and the miRNA assay used.** Mann-Whitney U results for data presented in Fig 2B of the manuscript showing differences within (A) samplings for each miRNA assay and (B) within miRNA assays.

(TIF)

**S7 Fig. Mann-Whitney U results for the Ct values (Fig 2C of the manuscript) highlighting the division of the participants upon miRNA expression.** Statistical analysis for data in Fig 2C showing statistical differences within participants (A) when averaging the six miRNAs and (B) within hsa-miR-103a-3p. We note that in panel A, participant P7 and participant P10 do not clearly belong to a group but rather are adjacent to a group, sharing partial significance.

(TIF)

**S8 Fig. Samplings presented higher variance than participants, demonstrating higher variability between the ten participants than between the four samplings for all six miRNA assays used in this study.**

(TIF)

**S9 Fig. Dose dependent responses of the six miRNAs assays demonstrate exponential behaviour.** Ct values for participant P2 (A), P6 (B) and P7 (C). (D) Average Ct values of the 3 participants with respect to the miRNA assay at different concentrations of total extracted small RNA. Error bars show the standard error of the mean.

(TIF)

**S10 Fig. Homogeneity in RT-qPCR efficiency of miRNA assays for participants P2 and P7 contrary to participant P6.** (A) RT-qPCR efficiencies calculated from the dose dependent data presented in S5 Fig. Mann-Whitney U results for panel A with respect to participants (B) and miRNA assay (C).

(TIF)

**S11 Fig. The dilution factor required to achieve 50 ng of small RNA input does not affect RT-qPCR efficiency of the six miRNA assays.** As for S4 Fig, two inputs concentrations were used, 1  $\mu$ L (circle) or 50 ng (cross). Again, all 50 ng values have been normalized with respect to the RNA concentration at 1  $\mu$ L (dilution factor) and all Ct values have been shifted with respect to the 1  $\mu$ L value. Although in this case the miRNA concentration may or not be heterogeneous, a linear behaviour is still obtained because we are observing the linear behaviour on the Ct value ( $\Delta Ct$ ) due to the dilution effect (log dilution factor).  $\Delta Ct$  values =  $Ct^{50ng} - Ct^{1\mu L}$ .

(TIF)

**S12 Fig. The limit of detection (LOD) and quantification (LOQ) differ for the six miRNA RT-qPCR assays.** Individual plots for data shown in Fig 3A of the manuscript, demonstrating high sensitivity (LOD of 1 copy/ $\mu$ L) for all six assays. Regression line calculated within LOQ and Ct saturation point (*i.e.* Ct value = 5). Points represent the mean value and error bars



depict the standard error of the mean. The grey region delimits the quantification region.  
(TIF)

**S13 Fig. RT-qPCR efficiency for the six miRNA assays analysing their synthetic targets.**

Data from [S12 Fig](#) was used to calculate efficiencies.

(TIF)

**S14 Fig. High cross-detection among the six miRNA assays used in this study.** Cross detections among miRNA assays when using (A)  $10^9$  copies/ $\mu$ L and (B)  $10^5$  copies/ $\mu$ L of synthetic targets. The absence of circles indicate no detection or Ct values = 35. This data was used to calculate the  $\Delta$ Ct values in [Fig 4B & 4C](#) of the manuscript.

(TIF)

**S15 Fig. The spike-in of synthetic hsa-lest-7a-5p miRNA decreases the Ct Values of extracted small RNA samples.** Data used to calculate the  $\Delta$ Ct values in [Fig 5](#) of the manuscript.

(TIF)

**S16 Fig. Heatmaps demonstrating the variability in sequence homology between the six miRNAs assessed in this study.** Sequence homology calculated by (A) number of homologous nucleotides and (B) percentage homology.

(TIF)

**S1 Table. The miRNAs chosen in this study.** Two similar pairs of miRNAs were included in this study, where they only differentiated by a single nucleotide (red font for the first pair and blue for the second pair).

(DOCX)

**S2 Table. Average RT-qPCR Ct values and the standard error of the mean of the four sampling points for each miRNAs assays assessed on the 10 participants.**

(DOCX)

**S3 Table. Dilution factors required to achieve 50 ng for the RT-qPCR reaction for the 10 participants and their 4 sampling points.**

(DOCX)

**S4 Table. Limit of detection (LOD) and limit of quantification (LOQ) for the six miRNA assays used in this study.**

(DOCX)

**S1 Dataset.**

(XLSX)

## Acknowledgments

The authors would like to thank all participants who made this study possible. We sincerely thanks Victor Petit, CEO of the company SkillCell for all the insightful discussion and comments from the beginning till the manuscript writing process. We thanks the CNRS for being the promotor of the study.

## Author Contributions

**Conceptualization:** Marc Van Der Hofstadt, Franck Molina, Thi Nhu Ngoc Van.

**Data curation:** Marc Van Der Hofstadt, Thi Nhu Ngoc Van.

**Formal analysis:** Marc Van Der Hofstadt, Thi Nhu Ngoc Van.

**Funding acquisition:** Franck Molina.

**Investigation:** Marc Van Der Hofstadt, Anna Cardinal, Morgane Lepeltier, Jérémy Boulestreau, Alimata Ouedraogo, Malik Kahli, Pierre Champigneux, Laurence Molina, Franck Molina, Thi Nhu Ngoc Van.

**Methodology:** Marc Van Der Hofstadt, Thi Nhu Ngoc Van.

**Project administration:** Franck Molina.

**Resources:** Franck Molina.

**Supervision:** Franck Molina, Thi Nhu Ngoc Van.

**Validation:** Marc Van Der Hofstadt, Thi Nhu Ngoc Van.

**Visualization:** Thi Nhu Ngoc Van.

**Writing – original draft:** Marc Van Der Hofstadt, Thi Nhu Ngoc Van.

**Writing – review & editing:** Anna Cardinal, Morgane Lepeltier, Jérémy Boulestreau, Alimata Ouedraogo, Malik Kahli, Pierre Champigneux, Laurence Molina, Franck Molina, Thi Nhu Ngoc Van.

## References

1. Lee YH, Wong DT. Saliva: an emerging biofluid for early detection of diseases. *Am J Dent*. 2009 Aug; 22(4):241–8. PMID: [19824562](https://pubmed.ncbi.nlm.nih.gov/19824562/)
2. Nonaka T, Wong DTW. Saliva Diagnostics. *Annual Rev Anal Chem*. 2022 Jun 13; 15(1):107–21. <https://doi.org/10.1146/annurev-anchem-061020-123959> PMID: [35696523](https://pubmed.ncbi.nlm.nih.gov/35696523/)
3. Ostheim P, Tichý A, Sirak I, Davidkova M, Stastna MM, Kultova G, et al. Overcoming challenges in human saliva gene expression measurements. *Sci Rep*. 2020 Jul 7; 10(1):11147. <https://doi.org/10.1038/s41598-020-67825-6> PMID: [32636420](https://pubmed.ncbi.nlm.nih.gov/32636420/)
4. Yoshizawa JM, Schafer CA, Schafer JJ, Farrell JJ, Paster BJ, Wong DTW. Salivary Biomarkers: Toward Future Clinical and Diagnostic Utilities. *Clin Microbiol Rev*. 2013 Oct; 26(4):781–91. <https://doi.org/10.1128/CMR.00021-13> PMID: [24092855](https://pubmed.ncbi.nlm.nih.gov/24092855/)
5. Hindson J Salivary miRNA signature for ESCC. *Nat Rev Gastroenterol Hepatol* [Internet]. 2023 Aug 8 [cited 2023 Aug 16]; Available from: <https://www.nature.com/articles/s41575-023-00837-5> <https://doi.org/10.1038/s41575-023-00837-5> PMID: [37553499](https://pubmed.ncbi.nlm.nih.gov/37553499/)
6. Mavroudis I, Petridis F, Balmus IM, Ciobica A, Gorgan DL, Luca AC. Review on the Role of Salivary Biomarkers in the Diagnosis of Mild Traumatic Brain Injury and Post-Concussion Syndrome. *Diagnostics*. 2023 Apr 7; 13(8):1367. <https://doi.org/10.3390/diagnostics13081367> PMID: [37189468](https://pubmed.ncbi.nlm.nih.gov/37189468/)
7. Setti Pezzi, Viani Pertinhez, Cassi Magnoni, et al. Salivary MicroRNA for Diagnosis of Cancer and Systemic Diseases: A Systematic Review. *IJMS*. 2020 Jan 30; 21(3):907. <https://doi.org/10.3390/ijms21030907> PMID: [32019170](https://pubmed.ncbi.nlm.nih.gov/32019170/)
8. Saliminejad K, Khorram Khorshid HR, Soleymani Fard S, Ghaffari SH. An overview of microRNAs: Biology, functions, therapeutics, and analysis methods. *Journal Cellular Physiology*. 2019 May; 234(5):5451–65. <https://doi.org/10.1002/jcp.27486> PMID: [30471116](https://pubmed.ncbi.nlm.nih.gov/30471116/)
9. Esteller M. Non-coding RNAs in human disease. *Nat Rev Genet*. 2011 Dec; 12(12):861–74. <https://doi.org/10.1038/nrg3074> PMID: [22094949](https://pubmed.ncbi.nlm.nih.gov/22094949/)
10. Shang R, Lee S, Senavirathne G, Lai EC. microRNAs in action: biogenesis, function and regulation. *Nat Rev Genet*. 2023 Dec; 24(12):816–33. <https://doi.org/10.1038/s41576-023-00611-y> PMID: [37380761](https://pubmed.ncbi.nlm.nih.gov/37380761/)
11. Bayraktar R, Van Roosbroeck K, Calin GA. Cell-to-cell communication: microRNAs as hormones. *Molecular Oncology*. 2017 Dec; 11(12):1673–86. <https://doi.org/10.1002/1878-0261.12144> PMID: [29024380](https://pubmed.ncbi.nlm.nih.gov/29024380/)
12. Qiu J, Xu J, Zhang K, Gu W, Nie L, Wang G, et al. Refining Cancer Management Using Integrated Liquid Biopsy. *Theranostics*. 2020; 10(5):2374–84. <https://doi.org/10.7150/thno.40677> PMID: [32089746](https://pubmed.ncbi.nlm.nih.gov/32089746/)

13. Robotti M, Scebba F, Angeloni D. Circulating Biomarkers for Cancer Detection: Could Salivary microRNAs Be an Opportunity for Ovarian Cancer Diagnostics? *Biomedicines*. 2023 Feb 21; 11(3):652. <https://doi.org/10.3390/biomedicines11030652> PMID: 36979630
14. Bahn JH, Zhang Q, Li F, Chan TM, Lin X, Kim Y, et al. The Landscape of MicroRNA, Piwi-Interacting RNA, and Circular RNA in Human Saliva. *Clinical Chemistry*. 2015 Jan 1; 61(1):221–30. <https://doi.org/10.1373/clinchem.2014.230433> PMID: 25376581
15. Nemeth K, Bayraktar R, Ferracin M, Calin GA. Non-coding RNAs in disease: from mechanisms to therapeutics. *Nat Rev Genet* [Internet]. 2023 Nov 15 [cited 2024 Jan 26]; Available from: <https://www.nature.com/articles/s41576-023-00662-1>. <https://doi.org/10.1038/s41576-023-00662-1> PMID: 37968332
16. Di Pietro VO'Halloran P, Watson CN, Begum G, Acharjee A, Yakoub KM, et al. Unique diagnostic signatures of concussion in the saliva of male athletes: the Study of Concussion in Rugby Union through MicroRNAs (SCRUM). *Br J Sports Med*. 2021 Dec; 55(24):1395–404. <https://doi.org/10.1136/bjsports-2020-103274> PMID: 33757972
17. Hicks SD, Onks C, Kim RY, Zhen KJ, Loeffert J, Loeffert AC, et al. Diagnosing mild traumatic brain injury using saliva RNA compared to cognitive and balance testing. *Clinical and Translational Medicine* [Internet]. 2020 Oct [cited 2023 Feb 28];10(6). Available from: <https://onlinelibrary.wiley.com/doi/https://doi.org/10.1002/ctm2.197> PMID: 33135344
18. Ghaith HS, Nawar AA, Gabra MD, Abdelrahman ME, Nafady MH, Bahbah EI, et al. A Literature Review of Traumatic Brain Injury Biomarkers. *Mol Neurobiol*. 2022 Jul; 59(7):4141–58. <https://doi.org/10.1007/s12035-022-02822-6> PMID: 35499796
19. Romani C, Salviato E, Paderno A, Zanotti L, Ravaggi A, Deganello A, et al. Genome-wide study of salivary miRNAs identifies miR-423-5p as promising diagnostic and prognostic biomarker in oral squamous cell carcinoma. *Theranostics*. 2021; 11(6):2987–99. <https://doi.org/10.7150/thno.45157> PMID: 33456584
20. Chirshv E, Oberg KC, Ioffe YJ, Unternaehrer JJ. *Let-7* as biomarker, prognostic indicator, and therapy for precision medicine in cancer. *Clinical & Translational Med*. 2019 Dec; 8(1):e24.
21. Bendifallah S, Suisse S, Puchar A, Delbos L, Poilblanc M, Descamps P, et al. Salivary MicroRNA Signature for Diagnosis of Endometriosis. *J Clin Med*. 2022 Jan 26; 11(3):612. <https://doi.org/10.3390/jcm11030612> PMID: 35160066
22. Bendifallah S, Dabi Y, Suisse S, Delbos L, Spiers A, Poilblanc M, et al. Validation of a Salivary miRNA Signature of Endometriosis—Interim Data. *NEJM Evidence* [Internet]. 2023 Jun 27 [cited 2024 Apr 12];2(7). Available from: <https://evidence.nejm.org/doi/https://doi.org/10.1056/EVIDOa2200282> PMID: 38320163
23. Slota Booth. MicroRNAs in Neuroinflammation: Implications in Disease Pathogenesis, Biomarker Discovery and Therapeutic Applications. *ncRNA*. 2019 Apr 24; 5(2):35. <https://doi.org/10.3390/ncrna5020035> PMID: 31022830
24. Monfared YK, Honardoost M, Cea M, Gholami S, Mirzaei-Dizgah I, Hashemipour S, et al. Circulating salivary and serum miRNA-182, 320a, 375 and 503 expression levels in type 2 diabetes. *J Diabetes Metab Disord*. 2022 Jul 28; 21(2):1469–78. <https://doi.org/10.1007/s40200-022-01082-4> PMID: 36404826
25. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci USA*. 2007 Jan 30; 104(5):1604–9. <https://doi.org/10.1073/pnas.0610731104> PMID: 17242365
26. Hicks SD, Olympia RP, Onks C, Kim RY, Zhen KJ, Fedorchak G, et al. Saliva microRNA Biomarkers of Cumulative Concussion. *IJMS*. 2020 Oct 20; 21(20):7758. <https://doi.org/10.3390/ijms21207758> PMID: 33092191
27. Johnson JJ, Loeffert AC, Stokes J, Olympia RP, Bramley H, Hicks SD. Association of Salivary MicroRNA Changes With Prolonged Concussion Symptoms. *JAMA Pediatr*. 2018 Jan 1; 172(1):65. <https://doi.org/10.1001/jamapediatrics.2017.3884> PMID: 29159407
28. Sandmo SB, Matyasova K, Filipcik P, Cente M, Koerte IK, Pasternak O, et al. Changes in circulating microRNAs following head impacts in soccer. *Brain Injury*. 2022 Mar 21; 36(4):560–71. <https://doi.org/10.1080/02699052.2022.2034042> PMID: 35172120
29. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, et al. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci USA* [Internet]. 2012 Jul 31 [cited 2024 Jan 27];109(31). Available from: <https://pnas.org/doi/full/10.1073/pnas.1209414109>. PMID: 22753494
30. Ding L, Lan Z, Xiong X, Ao H, Feng Y, Gu H, et al. The Dual Role of MicroRNAs in Colorectal Cancer Progression. *IJMS*. 2018 Sep 17; 19(9):2791. <https://doi.org/10.3390/ijms19092791> PMID: 30227605
31. Shi R, Sun YH, Zhang XH, Chiang VL. Poly(T) Adaptor RT-PCR. In: Fan JB, editor. *Next-Generation MicroRNA Expression Profiling Technology* [Internet]. Totowa, NJ: Humana Press; 2012 [cited 2024

- Jan 29]. p. 53–66. (Methods in Molecular Biology; vol. 822). Available from: [https://link.springer.com/10.1007/978-1-61779-427-8\\_4](https://link.springer.com/10.1007/978-1-61779-427-8_4) PMID: 22144191
32. Gines G, Menezes R, Xiao W, Rondelez Y, Taly V. Emerging isothermal amplification technologies for microRNA biosensing: Applications to liquid biopsies. *Molecular Aspects of Medicine*. 2020 Apr; 72:100832. <https://doi.org/10.1016/j.mam.2019.11.002> PMID: 31767382
  33. Jet T, Gines G, Rondelez Y, Taly V. Advances in multiplexed techniques for the detection and quantification of microRNAs. *Chem Soc Rev*. 2021; 50(6):4141–61. <https://doi.org/10.1039/d0cs00609b> PMID: 33538706
  34. Chen C. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Research*. 2005 Nov 27; 33(20):e179–e179. <https://doi.org/10.1093/nar/gni178> PMID: 16314309
  35. Androvic P, Valihrach L, Elling J, Sjoback R, Kubista M. Two-tailed RT-qPCR: a novel method for highly accurate miRNA quantification. *Nucleic Acids Research*. 2017 Sep 6; 45(15):e144–e144. <https://doi.org/10.1093/nar/gkx588> PMID: 28911110
  36. Honda S, Kirino Y. Dumbbell-PCR: a method to quantify specific small RNA variants with a single nucleotide resolution at terminal sequences. *Nucleic Acids Res*. 2015 Jul 13; 43(12):e77–e77. <https://doi.org/10.1093/nar/gkv218> PMID: 25779041
  37. Nuzziello N, Liguori M. Seeking a standardized normalization method for the quantification of microRNA expression. *Muscle and Nerve* [Internet]. 2019 May [cited 2024 Jan 29]; 59(5). Available from: <https://onlinelibrary.wiley.com/doi/https://doi.org/10.1002/mus.26455> PMID: 30809823
  38. Donati S, Ciuffi S, Brandi ML. Human Circulating miRNAs Real-time qRT-PCR-based Analysis: An Overview of Endogenous Reference Genes Used for Data Normalization. *IJMS*. 2019 Sep 5; 20(18):4353. <https://doi.org/10.3390/ijms20184353> PMID: 31491899
  39. Faraldi M, Gomarasca M, Sansoni V, Perego S, Banfi G, Lombardi G. Normalization strategies differently affect circulating miRNA profile associated with the training status. *Sci Rep*. 2019 Feb 7; 9(1):1584. <https://doi.org/10.1038/s41598-019-38505-x> PMID: 30733582
  40. Di Pietro V, Porto E, Ragusa M, Barbagallo C, Davies D, Forcione M, et al. Salivary MicroRNAs: Diagnostic Markers of Mild Traumatic Brain Injury in Contact-Sport. *Front Mol Neurosci*. 2018 Aug 20; 11:290. <https://doi.org/10.3389/fnmoi.2018.00290> PMID: 30177873
  41. Hicks SD, Onks C, Kim RY, Zhen KJ, Loeffert J, Loeffert AC, et al. Refinement of saliva microRNA biomarkers for sports-related concussion. *Journal of Sport and Health Science*. 2021 Aug; S209525462100096X. <https://doi.org/10.1016/j.jshs.2021.08.003> PMID: 34461327
  42. Bianchi F, Nicassio F, Marzi M, Belloni E, Dall'Olio V, Bernard L, et al. A serum circulating miRNA diagnostic test to identify asymptomatic high-risk individuals with early stage lung cancer. *EMBO Mol Med*. 2011 Aug; 3(8):495–503. <https://doi.org/10.1002/emmm.201100154> PMID: 21744498
  43. Redshaw N, Wilkes T, Whale A, Cowen S, Huggett J, Foy CA. A comparison of miRNA isolation and RT-qPCR technologies and their effects on quantification accuracy and repeatability. *BioTechniques*. 2013 Mar; 54(3):155–64. <https://doi.org/10.2144/000114002> PMID: 23477383
  44. Urbizu A, Arnaldo L, Beyer K. Obtaining miRNA from Saliva—Comparison of Sampling and Purification Methods. *IJMS*. 2023 Jan 25; 24(3):2386. <https://doi.org/10.3390/ijms24032386> PMID: 36768706
  45. Zyrina NV, Antipova VN, Zheleznaya LA. *Ab initio* synthesis by DNA polymerases. *FEMS Microbiol Lett*. 2014 Feb; 351(1):1–6.
  46. Tan E, Erwin B, Dames S, Ferguson T, Buechel M, Irvine B, et al. Specific versus Nonspecific Isothermal DNA Amplification through Thermophilic Polymerase and Nicking Enzyme Activities. *Biochemistry*. 2008 Sep 23; 47(38):9987–99. <https://doi.org/10.1021/bi800746p> PMID: 18729381
  47. Urtel G, Van Der Hofstadt M, Galas JC, Estevez-Torres A. rEXPAR: An Isothermal Amplification Scheme That Is Robust to Autocatalytic Parasites. *Biochemistry*. 2019 Jun 11; 58(23):2675–81. <https://doi.org/10.1021/acs.biochem.9b00063> PMID: 31074259
  48. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*. 2009 Apr 1; 55(4):611–22. <https://doi.org/10.1373/clinchem.2008.112797> PMID: 19246619