

Evaluation of RNA isolation methods for microRNA quantification in a range of clinical biofluids.

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Abstract

BACKGROUND Extracellular microRNAs (miRNAs), released from cells into biofluids, have emerged as promising biomarkers for diagnostic and prognostic purposes. For the analysis of these cell-free miRNAs in various biofluids by RT-qPCR, several RNA isolation methods are available. However, not all methods are equally suitable for different biofluids. The aim of this study is to evaluate the potential of different RNA isolation methods in a range of clinical biofluid samples.

METHODS Total RNA was isolated from serum, urine, bile, and graft preservation fluid (perfusate) using four different protocols: phenol-chloroform extraction in combination with a precipitation carrier, and three different column-based isolation methods. Co-purification of heparin, a known RT-qPCR inhibitor, was assessed using heparinase I during cDNA synthesis. Synthetic miRNAs, spiked-in during RNA workup (cel-miR-39) or during cDNA synthesis (cel-miR-54), and endogenous miRNAs were quantified using RT-qPCR.

RESULTS Recovery of cel-miR-39 significantly differed between methods with miRNeasy columns providing the best overall recovery in the four biofluids tested, as was also observed for endogenous miRNAs. Contamination of RNA with heparin differed between sample type and isolation method, and could be counteracted using heparinase I. Other co-isolated RT-qPCR inhibitors were not identified, except for biliverdin which co-isolated from some bile samples with one of the methods.

CONCLUSIONS For reliable measurements of miRNA-based biomarkers in biofluids, optimization of RNA isolation procedures is recommended as methods can differ in miRNA detection and co-purification of RT-qPCR inhibitory compounds. Heparinase I treatment confirmed that heparin appeared to be the major RT-qPCR-inhibiting compound but also biliverdin, co-isolated from bile, could interfere with detection.

Introduction

Small non-coding RNAs have been identified in both prokaryotic and eukaryotic organisms where they participate in a wide range of regulatory events (1). MicroRNAs (miRNAs) are a family of non-coding RNAs of approximately 18–24 nucleotides in length that are essential for post-transcriptional regulation of gene expression. With an estimated 60% of all genes being post-transcriptionally regulated by these molecules, miRNAs play an important role in many fundamental, biological processes (2, 3). Over 2500 different human miRNAs have been deposited in publicly available databases like miRBase (4), but likely still new ones will be identified (5). The observations that miRNA profiles are frequently altered during cellular development and pathology, indicate an important role in both malignant and non-malignant diseases (6). In addition, many miRNAs are also expressed in a tissue- or organ-specific manner, suggesting that miRNAs likely have high specificity and are applicable as biomarkers.

The discovery of disease-related variations of miRNAs in for instance blood or urine highlights their potential as minimal or non-invasive biomarkers (7). A plethora of studies were initiated in which cell-free miRNAs in serum or plasma were explored as biomarkers for disease diagnosis, prognosis and

monitoring of treatment responses. Although initially explored in cancer (8), changes in serum/plasma miRNA composition have also been observed for many other diseases, including cardiovascular, neurodegenerative and liver diseases (9–12). As extracellular miRNAs can reflect disease progression and treatment effects, they can also reflect tissue injury and graft outcome in the setting of organ transplantation (9, 13–16). Subsequent studies identified miRNAs in virtually all body fluids including breast milk, saliva, urine, cerebrospinal fluid, semen and bile as well as some non-bodily biofluids like organ preservation fluid (perfusate), broncho-alveolar lavage fluid and peritoneal dialysis effluent (17–22).

Two important properties of potential biomarkers are (i) ease of attainment of clinical samples, and (ii) robustness and ease of the detection assay. With the discovery of miRNAs and the presence of these molecules in biofluids, the condition for ease of attainment has been readily solved. Robustness of the miRNA assay is challenged at multiple levels as it requires stability during sampling, storage, and subsequent processing, and must also be executed in a consistent and reproducible fashion with as little technical variation as possible (23). Whereas stability of miRNAs during sampling and storage has been confirmed (21, 24–27) and detection using validated commercial assays is widely available, the optimal isolation procedure for miRNAs from different biofluids remains less established. Several studies have been published which address the question of miRNA isolation (28–31) but often yield inconsistent or sometimes even controversial results, and do not consider the presence of co-purified inhibitory compounds like heparin (32, 33). Many methods and commercial kits are available for miRNA isolation of biofluids, but data on which method/kit is most suitable for which biofluid is still lacking. Therefore, the aim of this study is to determine the most robust and all-round miRNA isolation procedure for a number of human biofluids that were obtained in a clinical setting.

Materials And Methods

Sample collection and processing

Clinical samples were obtained from patients undergoing liver transplantation surgery performed at the Erasmus MC in Rotterdam, the Netherlands. Bile from liver grafts was collected and processed essentially as described previously (27). Graft preservation fluid (perfusate) samples were obtained during the back-table procedure. Upon arrival at the operating room, grafts were flushed *ex situ* with University of Wisconsin solution (Viaspan, Duramed Pharm Inc, Pomona, NY), followed by a flush of human albumin solution (Albuman human albumin 40 g/l, Sanquin, The Netherlands) just prior to implantation. The effluent of this second flush was collected and processed as described by Verhoeven et al (17). Blood and urine were obtained within 24 hours after surgery and processed immediately. Blood was collected in Vacutainer serum tubes (Becton Dickinson, Breda, The Netherlands), centrifuged at 18 °C for 10 min at 800 g to separate serum. Urine was collected as described previously (34). Cell-free material was stored at -20 °C until further use.

Drugs and reagents

Heparin used in this study was laboratory grade, and in-house manufactured by the hospital pharmacy (500 IU/mL). For co-purification analysis heparin was diluted in standard saline solution (0.7%) in a serial dilution.

RNA isolation

Total RNA was extracted using four different protocols; (RN) Qiagen miRNeasy kit (Qiagen, Venlo, The Netherlands), (QP) Qiazol (Qiagen, Venlo, The Netherlands) in combination with the precipitation carrier (dr. GenTLE, Takara, Kusatsu, Japan), (NG) NORGEN Total RNA isolation kit (Norgen biotek, Thorold, Canada), and (CU) miRCURY RNA isolation kit - biofluids (Exiqon, Vedbæk, Denmark). For QP, 2 × 100 µl of sample was lysed in 2 × 1 ml Qiazol as described by the manufacturer. After adding 200 µl chloroform, samples were centrifuged for 15 min, 12000 × *g* at 4 °C. 500 µl of the upper, aqueous, phase of both samples was transferred to one new collection tube. RNA was precipitated by subsequent addition of 100 µl 3M NaAc (pH 5.2), 10 µl of Dr. Gentle precipitation solution, and 1 ml iso-propanol, with vortexing after each addition. Samples were kept for 10 min at room temperature, followed by centrifugation for 10 min, 12000 × *g* at 4 °C. Pellets were washed with 1 ml 75% ethanol, mixed by vortexing and centrifuged for 5 min, 7500 × *g* at 4 °C. After a second wash, pellets were dried for 10 min at room temperature and RNA was dissolved in RNase free water. Isolation of RNA using miRNeasy, NORGEN, and miRCURY columns were executed as described in the manufacturer's guidelines. The main properties of these isolation methods are indicated in Table 1. Step-by-step protocols can be found in the Additional file 1. In all cases, samples were spiked with 200 amol of artificial *Caenorhabditis elegans* miR-39 (cel-miR-39, Sigma Aldrich, Zwijndrecht, The Netherlands) during the lysis procedure to monitor loss during workup and 100 amol cel-miR-54 (Sigma) during cDNA synthesis to detect residual heparin contamination and other PCR inhibiting compounds co-purified with RNA.

Table 1
Properties of RNA isolation methods

	RN	QP	NG	CU
Sample volume (µl)	200	2 × 100	200	200
Phenol extraction	Yes	Yes	No	No
Precipitation carrier	No	Yes	No	No
Column-based	Yes	No	Yes	Yes
(Elution) volume (µl)	30	30	50	50

Heparinase I treatment

5 µL of isolated total RNA was added to an RT reaction mixture containing 6 IU heparinase I (New England Biolabs, Ipswich, MA) and heparin degradation was obtained during the RT step for cDNA synthesis as described previously (34). heparinase I treatment is included unless indicated otherwise.

Reverse transcriptase and quantitative real-time polymerase chain reaction

cDNA was synthesized using the Taqman microRNA Reverse Transcription Kit (Applied Biosystems/Life technologies, Carlsbad, CA) as described previously (17), using 5 µL of isolated total RNA. cDNA was diluted to 100 µL with water and stored at -20 °C. PCR reactions were conducted on an Applied Biosystems StepONE plus real-time PCR machine (Applied Biosystems) according to the manufacturer's guidelines with 45 cycles of amplification. Reactions consist of 6 µL Taqman Universal PCR Master mix (Life technologies), 0.5 µL miRNA specific primer, 0.5 µL sterile milliQ water and 5 µL of diluted cDNA. The mature sequences of miRNAs analyzed, both endogenous and synthetic, are summarized in Table 2. Threshold levels for quantification of PCR results were manually set at 0.25 for all microRNA assays, and the upper Cq limit for reliable detection was set at 35 cycles. Heparinase I was included in the RT reaction unless indicated otherwise.

Table 2
Mature miRNA sequences and assays

miRNA	Mature sequence	Assay ID*
cel-miR-39	UCACCGGGUGUAAAUCAGCUUG	000200
cel-miR-54	UACCCGUAUAUCUUCAUAAUCCGAG	001361
hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA	000397
hsa-miR-30e	UGUAAACAUCUUGACUGGAAG	002223
hsa-miR-92a	UAUUGCACUUGUCCCGGCCUGU	000431
hsa-miR-122	UGGAGUGUGACAAUGGUGUUUG	002245
hsa-miR-222	AGCUACAUCUGGCUACUGGGU	002276

*: Assay identification number as provided by ThermoFisher for the detection of individual miRNAs.

Statistical analysis

Levels of miRNAs as detected by PCR were converted to relative detection levels using the following equation: relative detection levels = $2^{(-Cq)} \times 10^9$. Wilcoxon matched paired tests, Mann-Whitney U tests and nonlinear regression for dose-response analyses were performed using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA). *p*-values < 0.05 were considered significant.

Results

Evaluation of miRNA recovery during isolation for different biofluids

The robustness of an assay is an essential parameter for miRNA detection. To test the miRNA recovery, the levels of a synthetic spiked-in miRNA (cel-miR-39) in six matched human serum, urine, bile and

perfusate samples were measured using the four aforementioned methods. The direct output of the PCR results showed that the median Cq values were the lowest for the RN method in all 4 analyzed biofluids (23.15, 23.26, 22.3, and 22.85 for serum, urine, perfusate, and bile, respectively) and the most consistent, as determined by the smallest range, in urine, perfusate, and bile (23.01–24.45, 21.76–22.59, and 22.21–23.62, respectively). To determine the relative improvement of detection using the RN method in comparison to methods QP, NG, and CU, relative detection levels were converted to percentages with the mean of the RN method for each biofluid set to 100% (Fig. 1). The mean recovery of cel-miR-39 from serum using the RN method was significantly better than the recovery using the QP and CU method with levels (mean% \pm SEM) reaching only 33.8% \pm 14.4 and 45.8% \pm 6.1, respectively. Recovery from serum using the NG method was also less than half of the RN method (36.1% \pm 6.9), but not significantly different from RN (Fig. 1A). The recovery improvement of cel-miR-39 from urine samples was even more pronounced when RNA was isolated from urine samples. The RN method performed significantly better than methods QP, NG, and CU, with recovery levels of 32.5% \pm 9.3, 41.5% \pm 8.9, and 10.3% \pm 3.3, respectively (Fig. 1B). The significantly better performance of the RN method was also observed for RNA isolated from perfusate with recovery levels reaching only 29.8% \pm 4.4, 10.8% \pm 2.3, and 22.2% \pm 5.8 for QP, NG, and CU, respectively, compared with the RN method (Fig. 1C). Although recovery of RNA from bile was better with method RN, the levels were not significantly improved when compared with the QP and CU method (66.6% \pm 15.5 and 56.1% \pm 14.0). Recovery from bile using the NG method, however, was significantly lower (1.6% \pm 0.3) (Fig. 1D).

The effect of the different RNA isolation methods on the yield of endogenous miRNA present in the four biofluids is shown in Fig. 2. For serum, miR-122, miR-222 and miR-21 were determined (Fig. 2A). For urine, miR-30a and miR-92e were determined (Fig. 2B), for the biofluids bile and PF both miR-122 and miR-222 were determined (Fig. 2C, D). Overall the same recovery trend were seen with the endogenous miRNAs as with spiked-in miRNA cel-miR-39

Isolation methods differ in the co-purification of heparin

As shown in multiple studies, anti-coagulants could strongly affect the analysis of miRNAs for biomarker discovery. Heparin contamination is known to negatively influence the quantification of miRNAs in blood samples and urine, but also in perfusate. To circumvent this specific inhibition, the use of heparinase I in cDNA synthesis was propagated (34–36). To assess any possible differences in co-isolation of heparin between the different methods, four perfusate samples with proven heparin contamination were selected from a previous study (36). Total RNA was isolated using methods QP, RN, and NG and RT-qPCR was performed in the absence (-) or presence (+) of heparinase I. The RT-qPCR results for cel-miR-54 in the presence or absence of heparinase I showed a clear increase in relative detection levels for methods RN and QP, while the improvement with RNA isolated with the NG method is only marginal (Fig. 3A). Results for recovery of cel-miR-39 were presented as relative detection levels in Fig. 3B. Co-purification of heparin was most prominent in RNA isolated using methods QP and RN, where treatment with heparinase I resulted in a mean increase of the relative detection levels of 804- and 236-fold, respectively. Heparinase I treatment during cDNA synthesis of RNA isolated using the NG method, on the other hand, only resulted in a 2-fold improvement (Fig. 3B). As already shown previously, relative detection levels after heparinase I

treatment were the highest in RNA isolated using method RN. We also tested the effect of heparin contamination on endogenous miRNAs miR-122 and miR-222, two miRNAs that have shown to be well detectable in perfusate samples (17). Again, detection of miRNAs clearly improved when heparinase I treatment was applied to RNA isolated through methods QP and RN. RNA isolated with method NG again showed little, if any, evidence of heparin co-purification as no improvement was observed after treatment with heparinase I for miR-122 and miR-222 (Fig. 3C and D). The RT-qPCR results for cel-miR-54 in the presence or absence of heparinase I also showed that no additional inhibitory compounds were isolated specific for one of these 3 methods as mean relative detection levels after heparinase I treatment for methods RN, QP, and NG were 856, 820, and 825, respectively (Fig. 3A).

As the RN method resulted in the best recovery of spiked-in cel-miR-39 and the NG method showed the strongest insensitivity for co-purification of heparin, these methods were combined. Lysis/denaturation of one method was combined with the columns of the other method and vice versa. This experiment did not result in a hybrid method that showed recovery levels of the RN method with the heparin insensitivity of the NG method as is shown in Supplemental Fig. 1.

The RNA isolation procedure NG is not completely insensitive to heparin contamination

Initially considered as a method that could isolate RNA without heparin contamination, results suggested that a certain level of heparin contamination could still be detected in the RNA samples isolated with the NG method in the absence of heparinase I (Fig. 3). To follow up on this observation and determine to what extent and to what level heparin contamination is present in RNA samples isolated with the NG method, clean (un-used) UW solution was spiked the standard amount of cel-miR-39 in combination with increasing amounts of heparin. RNA was isolated using either the NG or the RN method, and RT-qPCR detection of cel-miR-39 and cel-miR-54 was performed in the absence of heparinase I. For both cel-miR-54 (Fig. 4A) and cel-miR-39 (Fig. 4D) reduction of relative detection levels with increasing amounts of heparin was very similar between methods RN and NG, suggesting that the latter procedure does not prevent heparin contamination. To determine whether the observed discrepancy was caused by components only present in body fluids, urine and serum samples from four patients were pooled and also spiked with increasing amounts of heparin. Spiked-in cel-miR-39 and cel-miR-54 were determined as described for the clean UW samples. Nonlinear regression (curve fit) comparison of dose-response data on log transformed, normalized duplicates was used for statistical analysis. RNA isolated with the NG method from both serum and urine appeared significantly less contaminated with heparin when compared to RNA isolated with the RN method ($p < 0.0001$). This was determined for both for the spike-in added during the work-up procedure (Fig. 4B and C) as well as for the spike-in added during the cDNA synthesis procedure (Fig. 4E and F). This pointed to the presence of components in body fluids that prevent co-isolation of heparin with the NG method.

Evaluation of co-purification of PCR-inhibiting compounds other than heparin

RT-qPCR analyses are prone for interference by compounds that co-purify with RNA (37). Besides heparin, other interfering compounds can also be co-purified which cannot be counteracted by heparinase I treatment. As already shown, a substantial variation was observed in the detection levels of cel-miR-39,

hinting towards the presence of non-heparinous compounds. Therefore the RNA samples used previously (obtained from serum, urine, perfusate and bile of six patients), were spiked with cel-miR-54 and cDNA synthesis and RT-qPCR analysis performed. Relative detection levels of cel-miR-54 in serum were not significantly different between methods RN, QP, NG, and CU with values ranging between 1157–1444, 1207–1465, 1122–1473 and 802–1441 (Fig. 5A). Results for urine were comparable with respective values ranging from 1140–1457, 1310–1468, 1309–1637 and 1012–1591, respectively (Fig. 5B). Results for perfusate were also not different between the four different methods with values ranging between 1408–2141, 1406–2181, 1471–2082 and 1049–2167 for RN, QP, NG and CU, respectively (Fig. 5C). As the reference values of cel-miR-54, indicated by the red line in Fig. 5, were 1358, 1571 and 2101 for serum, urine and perfusate, respectively, the co-purification of other PCR-inhibiting compounds from these biofluids seemed almost absent.

Contrary to serum, urine and perfusate, levels of cel-miR-54 spiked in RNA samples from bile isolated showed a larger extent of variation. Relative detection levels range from 1373–1917, 1288–1958, and 1515–2165 for the RN, QP, and NG method, but 32–1884 for the CU method (Fig. 5D). This increased range for cel-miR-54 levels in RNA from bile could be attributed to one sample that showed a distinct green discoloration. To follow up on this observation, the number of bile samples analyzed was increased to 10 and cel-miR-39 and cel-miR-54 levels were again determined. Four out of 10 RNA samples showed this discoloration after isolation (Fig. 6A), and this phenomenon was associated with lower relative detection levels of both cel-miR-39 and cel-miR-54, which was probably caused by co-isolation of biliverdin when the CU method was used (Fig. 6B).

Discussion

In this study, we evaluated the efficiency of four different methods to isolate miRNAs from some very unique and diverse biofluids, explored their use in more common biofluids like serum and urine, and determined their sensitivity towards contamination by the anti-coagulant heparin, being a clinically relevant and frequently used drug. To our knowledge, this is the first report that describes a systematic approach to determine the most optimal method for a range of specific biofluids. In addition, we also determined their applicability on biofluids more relevant for biomarker discovery and measurement, and in that way identify the most robust method for general use. Many different methods and kits were developed that allow for the isolation of total RNA without the loss of small RNA. From our results, we concluded that the miRNeasy kit was the most effective in isolating small RNA molecules from two very distinct biofluids, bile and perfusate as measured by RT-qPCR, while retaining qualitative and quantitative good levels of detection in serum and urine.

Analyzing the results for each individual method, the recovery of cel-miR-39 displayed a comparable pattern as we observed with the endogenous miRNAs (Figs. 1 and 2). Identification of robust controls for data normalization remains a challenge when biofluids are considered for miRNA biomarker discovery the use of a spike-in is the bare minimum. Although it was previously shown that the use of spike-in miRNAs in RNA isolation from serum and plasma resulted in high variability between samples and

should not be used as a normalizer for RT-qPCR analysis, our study confirmed that cel-miR-39 recovery does provide a good indication for loss of RNA during workup when comparing recovery levels from different procedures. Especially useful for less common biofluids like bile and, preservation fluids and, to a lesser extent, in urine, where the identification of a suitable normalizing miRNA will even be more difficult than it is in serum and plasma. Recovery of cel-miR-39 when spiked in serum or urine also turned out most optimal with the RN method. Although we didn't include any method that is serum/plasma or urine specific, our data suggests that RNA, isolated with the standard, more general, miRNA isolation methods, can also provide good downstream results. This clearly shows that more general methods for RNA isolation are robust and applicable for a wide range of biofluids.

RT-qPCR is the preferred choice to measure miRNA levels in biofluids, as other methods are, at present, less sensitive. Other downstream platforms might require other miRNA isolation methods to obtain more reliable data. Srinivasan et al. did an extensive analysis of ten methods in five different biofluids and analyzed the RNA samples using small RNA-seq. Their study showed that the CU method was considered the most reproducible for the bile samples that were analyzed, however with low complexity, suggesting further investigation was warranted (38). Recently, Godoy et al. performed a comprehensive RNA-seq analysis for which they also used the miRNeasy method for RNA isolation (39), suggesting that this method can be applied for various downstream applications.

Anticoagulants are amongst the most notorious inhibitors of RT-qPCR analysis. Heparin contamination, however, is not only a confounding factor in the analysis of plasma or serum, but can also play an important role in other biofluids like urine and preservation fluids as was previously shown (34, 36). This phenomenon, as also shown in this study, appeared independent of the RNA isolation methods used. Co-purification of heparin occurred at a variable level, in every method we analyzed, and the use of heparin-degrading enzymes is, therefore, recommended. Levels of cel-miR-54 were shown to be very consistent between all serum, urine and perfusate samples when heparinase I treatment was included in the RT-qPCR analysis, and suggests that other inhibitory compounds, present in these three biofluids, were not co-purified or only present in very small concentrations. The presence of inhibitory compounds other than heparin can never be excluded as was shown in RNA samples isolated from bile using method CU, where the presence of biliverdin strongly affects qPCR detection. This clearly shows that the possibility of co-purification of inhibitory components is both biofluid and isolation method dependent and attention should be paid to their presence.

Despite the attention on RT-qPCR inhibiting compounds, our results provided evidence that there are also components present in biofluids that can actually reduce the detrimental effect of heparin on downstream applications like RT-qPCR. Where spiked-in miRNAs in a synthetic solution, such as fresh UW, showed no clear difference in kinetics between the RN and NG method, serum or urine contain compounds that affect the co-elution of heparin when the NG method was used. These varying results do emphasize the need for standard heparinase I treatment to avoid conflicting results.

Throughout this study, we focused our attention on biofluids that are related to liver transplantation and that have shown to be useful in identifying and applying miRNAs as biomarkers for complications after this surgical procedure. Although a limited set, the biofluids used in this study represent, perhaps, not only the most hostile conditions to RNA, but also the most challenging to isolate RNA from. It should also be noted that we used a selection of RNA isolation methods. These are, however, among the more frequently used methods and therefore represent methods that are applied in many scientific papers. The fact that we didn't include methods that are specific for a single biofluid, was intentional, as we evaluated robustness and general applicability.

Conclusions

In this report, we compared four different methods for total RNA isolation on a diverse set of biofluids. We observed that treatment of isolated RNA with heparinase I is essential, and methods that have a more general character are also suitable for biofluids, like serum and urine, for which specialized isolation methods have been developed. In addition, not only heparin gets co-purified with the methods presented in this study, but other substances that inhibit RT-qPCR results are also identified.

List Of Abbreviations

miR/miRNA: microRNA

RT-qPCR: reverse transcription quantitative real-time PCR

cel: *Caenorhabditis elegans*

hsa: *Homo sapiens*

QP: Classic phenol-chloroform extraction with Dr.GenTLE as a precipitation carrier

RN: miRNeasy mini kit

NG: NORGEN total RNA purification kit

CU: miRCURY RNA Isolation Kit – Biofluids

Declarations

Ethics approval and consent to participate

Collection of peri-transplant materials from hospitalized individuals was approved by the Medical Ethical Council of the Erasmus MC and all participants provided informed consent for the use of these materials for medical research.

Consent for publication

Not applicable

Availability of data and materials

Data used and/or analyzed in this study are available from the corresponding author upon request

Competing interests

No competing interests to declare

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Author's contributions

HPR performed the experiments, analyzed and interpreted the data, and wrote the manuscript. JNMIJ contributed to the study design, provided patient materials and reviewed the manuscript. LJWL contributed to the study design, supervised the study, and reviewed the manuscript. All authors approved the current manuscript.

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References

1. Storz G. An expanding universe of noncoding RNAs. *Science*. 2002;296(5571):1260-3.
2. Ambros V. The functions of animal microRNAs. *Nature*. 2004;431(7006):350-5.
3. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009;19(1):92-105.
4. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014;42(Database issue):D68-73.
5. Fehlmann T, Backes C, Kahraman M, Haas J, Ludwig N, Posch AE, et al. Web-based NGS data analysis using miRMaster: a large-scale meta-analysis of human miRNAs. *Nucleic Acids Res*. 2017;45(15):8731-44.
6. Alvarez-Garcia I, Miska EA. MicroRNA functions in animal development and human disease. *Development*. 2005;132(21):4653-62.
7. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;18(10):997-1006.
8. Wang J, Zhang KY, Liu SM, Sen S. Tumor-associated circulating microRNAs as biomarkers of cancer. *Molecules*. 2014;19(2):1912-38.

9. Amrouche L, Rabant M, Anglicheau D. MicroRNAs as biomarkers of graft outcome. *Transplant Rev (Orlando)*. 2014;28(3):111-8.
10. Loosen SH, Schueller F, Trautwein C, Roy S, Roderburg C. Role of circulating microRNAs in liver diseases. *World J Hepatol*. 2017;9(12):586-94.
11. Viereck J, Thum T. Circulating Noncoding RNAs as Biomarkers of Cardiovascular Disease and Injury. *Circ Res*. 2017;120(2):381-99.
12. Zi Y, Yin Z, Xiao W, Liu X, Gao Z, Jiao L, et al. Circulating MicroRNA as Potential Source for Neurodegenerative Diseases Biomarkers. *Mol Neurobiol*. 2015;52(3):1494-503.
13. Hamdorf M, Kawakita S, Everly M. The Potential of MicroRNAs as Novel Biomarkers for Transplant Rejection. *J Immunol Res*. 2017;2017:4072364.
14. Laterza OF, Lim L, Garrett-Engle PW, Vlasakova K, Muniappa N, Tanaka WK, et al. Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury. *Clin Chem*. 2009;55(11):1977-83.
15. Mas VR, Dumur CI, Scian MJ, Gehrau RC, Maluf DG. MicroRNAs as biomarkers in solid organ transplantation. *Am J Transplant*. 2013;13(1):11-9.
16. Zhou M, Hara H, Dai Y, Mou L, Cooper DK, Wu C, et al. Circulating Organ-Specific MicroRNAs Serve as Biomarkers in Organ-Specific Diseases: Implications for Organ Allo- and Xeno-Transplantation. *Int J Mol Sci*. 2016;17(8).
17. Verhoeven CJ, Farid WR, de Ruiter PE, Hansen BE, Roest HP, de Jonge J, et al. MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation. *J Hepatol*. 2013;59(6):1231-8.
18. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. *Clin Chem*. 2010;56(11):1733-41.
19. Chen J, Kam-Tao P, Kwan BC, Chow KM, Lai KB, Luk CC, et al. Relation between microRNA expression in peritoneal dialysis effluent and peritoneal transport characteristics. *Dis Markers*. 2012;33(1):35-42.
20. Kishore A, Navratilova Z, Kolek V, Novosadova E, Cepe K, du Bois RM, et al. Expression analysis of extracellular microRNA in bronchoalveolar lavage fluid from patients with pulmonary sarcoidosis. *Respirology*. 2018;23(12):1166-72.
21. Shigehara K, Yokomuro S, Ishibashi O, Mizuguchi Y, Arima Y, Kawahigashi Y, et al. Real-time PCR-based analysis of the human bile microRNAome identifies miR-9 as a potential diagnostic biomarker for biliary tract cancer. *PLoS One*. 2011;6(8):e23584.
22. Roest HP, Ooms LSS, Gillis AJM, JNM IJ, Looijenga LHJ, Dorssers LCJ, et al. Cell-free MicroRNA miR-505-3p in Graft Preservation Fluid Is an Independent Predictor of Delayed Graft Function After Kidney Transplantation. *Transplantation*. 2019;103(2):329-35.
23. Dellett M, Simpson DA. Considerations for optimization of microRNA PCR assays for molecular diagnosis. *Expert Rev Mol Diagn*. 2016;16(4):407-14.
24. Lankisch TO, Voigtlander T, Manns MP, Holzmann A, Dangwal S, Thum T. MicroRNAs in the bile of patients with biliary strictures after liver transplantation. *Liver Transpl*. 2014;20(6):673-8.

25. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008;105(30):10513-8.
26. Mraz M, Malinova K, Mayer J, Pospisilova S. MicroRNA isolation and stability in stored RNA samples. *Biochem Biophys Res Commun*. 2009;390(1):1-4.
27. Verhoeven CJ, Farid WR, Roest HP, Ramakrishnaiah V, de Ruiter PE, de Jonge J, et al. Polarized release of hepatic microRNAs into bile and serum in response to cellular injury and impaired liver function. *Liver Int*. 2016;36(6):883-92.
28. Brunet-Vega A, Pericay C, Quilez ME, Ramirez-Lazaro MJ, Calvet X, Lario S. Variability in microRNA recovery from plasma: Comparison of five commercial kits. *Anal Biochem*. 2015;488:28-35.
29. Eldh M, Lotvall J, Malmhall C, Ekstrom K. Importance of RNA isolation methods for analysis of exosomal RNA: evaluation of different methods. *Mol Immunol*. 2012;50(4):278-86.
30. El-Khoury V, Pierson S, Kaoma T, Bernardin F, Berchem G. Assessing cellular and circulating miRNA recovery: the impact of the RNA isolation method and the quantity of input material. *Sci Rep*. 2016;6:19529.
31. Monleau M, Bonnel S, Gostan T, Blanchard D, Courgnaud V, Lecellier CH. Comparison of different extraction techniques to profile microRNAs from human sera and peripheral blood mononuclear cells. *BMC Genomics*. 2014;15:395.
32. Moldovan L, Batte KE, Trgovcich J, Wisler J, Marsh CB, Piper M. Methodological challenges in utilizing miRNAs as circulating biomarkers. *J Cell Mol Med*. 2014;18(3):371-90.
33. Boeckel JN, Thome CE, Leistner D, Zeiher AM, Fichtlscherer S, Dimmeler S. Heparin selectively affects the quantification of microRNAs in human blood samples. *Clin Chem*. 2013;59(7):1125-7.
34. Roest HP, Verhoeven CJ, de Haan JE, de Jonge J, JN IJ, van der Laan LJ. Improving Accuracy of Urinary miRNA Quantification in Heparinized Patients Using Heparinase I Digestion. *J Mol Diagn*. 2016;18(6):825-33.
35. Plieskatt JL, Feng Y, Rinaldi G, Mulvenna JP, Bethony JM, Brindley PJ. Circumventing qPCR inhibition to amplify miRNAs in plasma. *Biomark Res*. 2014;2:13.
36. Verhoeven CJ, Selten JW, Roest HP, Farid WRR, de Ruiter PE, Hansen BE, et al. Corrigendum to "MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation" [*J Hepatol* 2013; 59:1231-1238]. *J Hepatol*. 2017.
37. Schrader C, Schielke A, Ellerbroek L, John R. PCR inhibitors - occurrence, properties and removal. *J Appl Microbiol*. 2012;113(5):1014-26.
38. Srinivasan S, Yeri A, Cheah PS, Chung A, Danielson K, De Hoff P, et al. Small RNA Sequencing across Diverse Biofluids Identifies Optimal Methods for exRNA Isolation. *Cell*. 2019;177(2):446-62 e16.
39. Godoy PM, Bhakta NR, Barczak AJ, Cakmak H, Fisher S, MacKenzie TC, et al. Large Differences in Small RNA Composition Between Human Biofluids. *Cell Rep*. 2018;25(5):1346-58

Figures

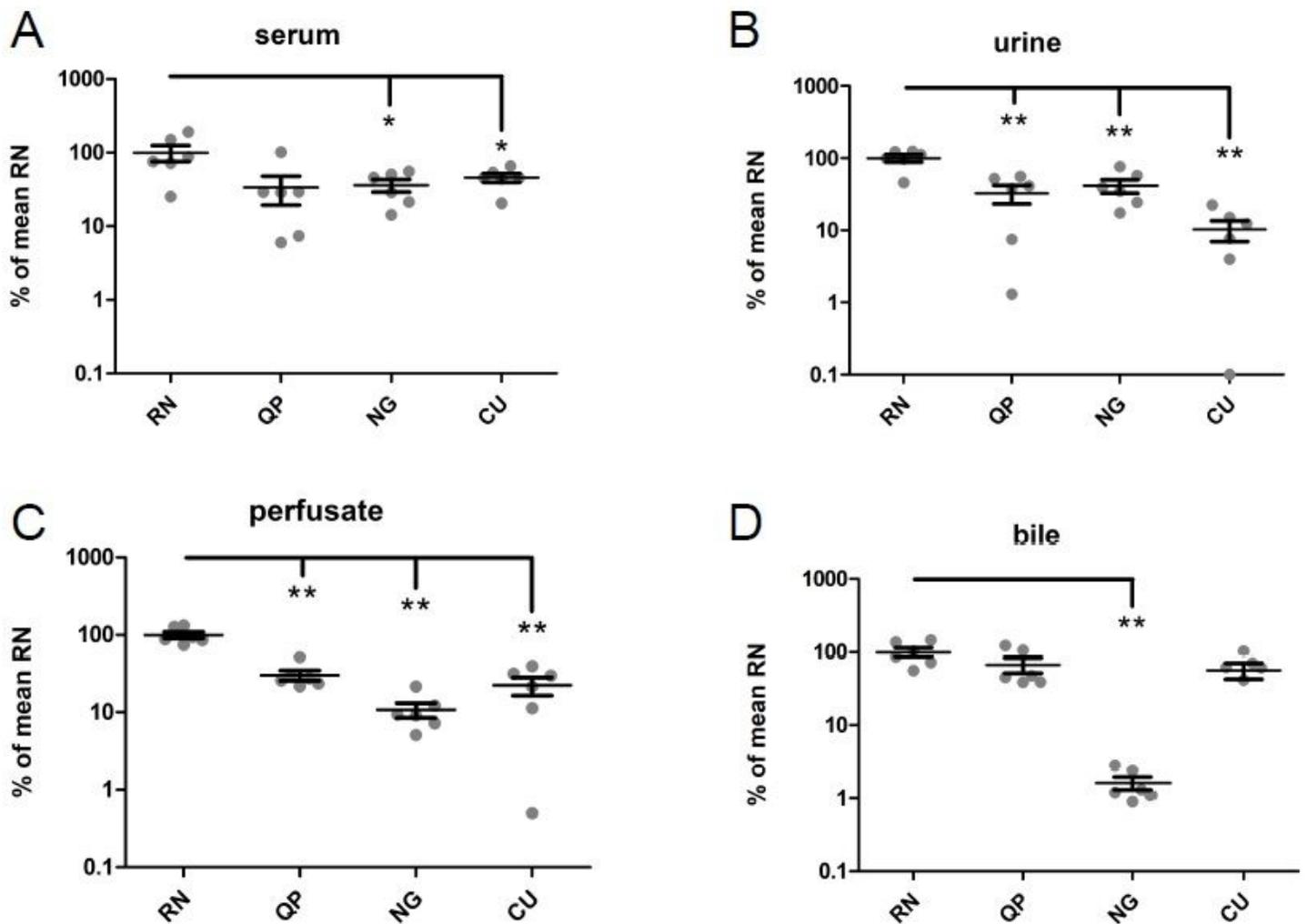


Figure 1

Relative detection levels of spiked-in cel-miR-39 in four biofluids. Levels of cel-miR-39, spiked during workup, were determined in serum (A), urine (B), perfusate (C), and bile (D) from six patients and their donor organs. Results are shown of four different methods and presented as scatter plots (mean \pm SEM). RN; Qiagen miRNeasy kit, QP; Qiazol in combination with the dr. GentLE precipitation carrier, NG; NORGEN Total RNA isolation kit, and CU; miRCURY RNA isolation kit - biofluids. The mean of the levels with the RN isolation method per liquid were set at 100% . Data are presented as mean \pm SEM. Overall the RN methods gave best recovery. *: $p < 0.05$, **: $p < 0.01$, Mann-Whitney U test.

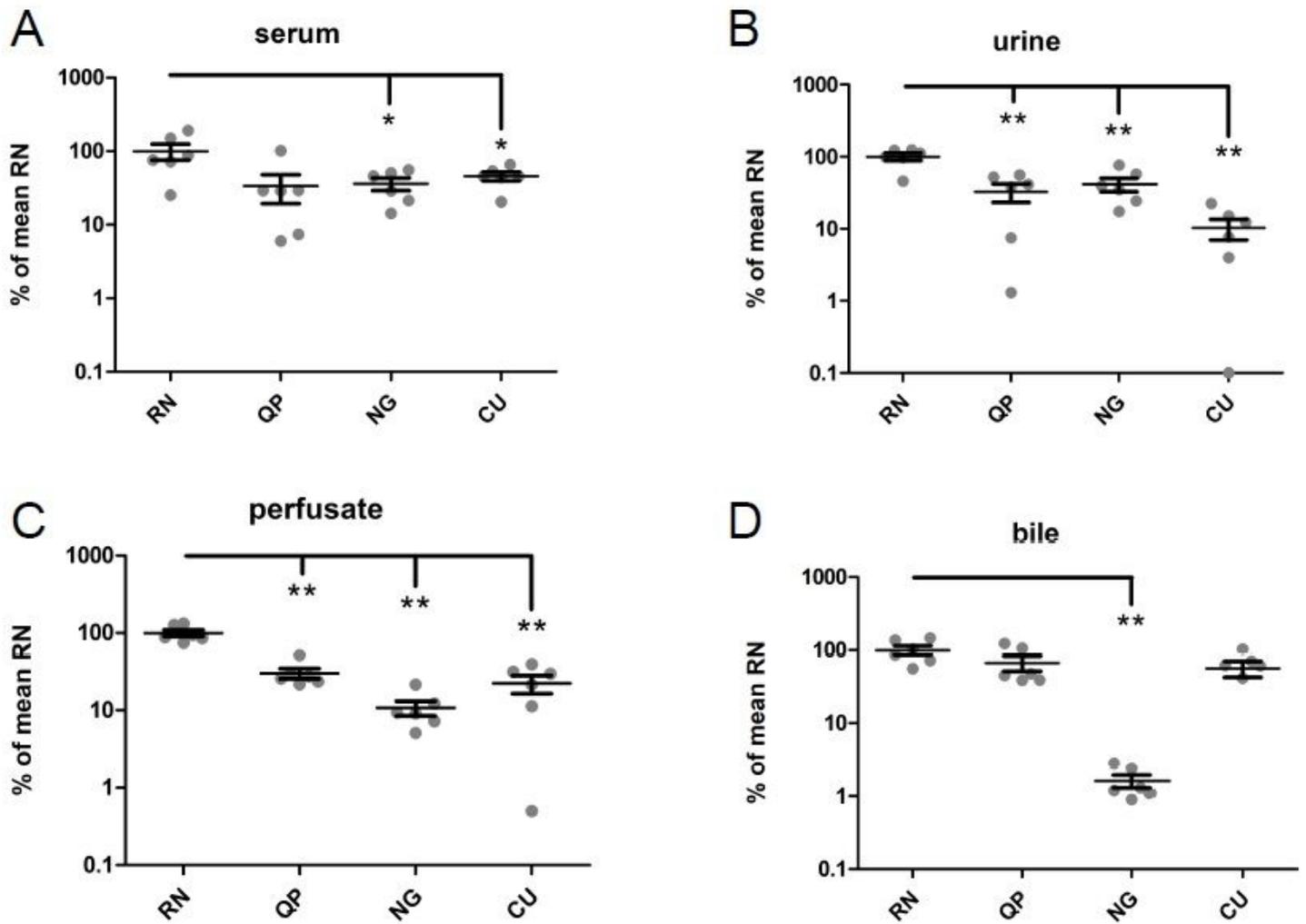
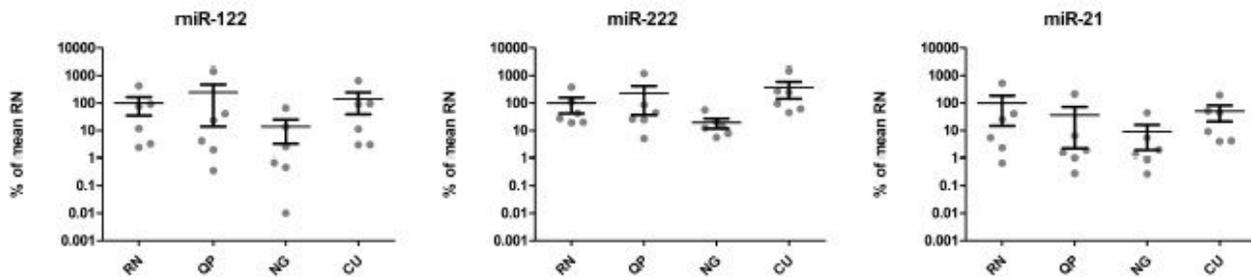


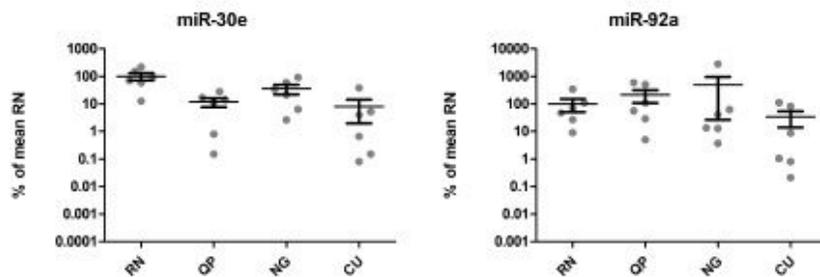
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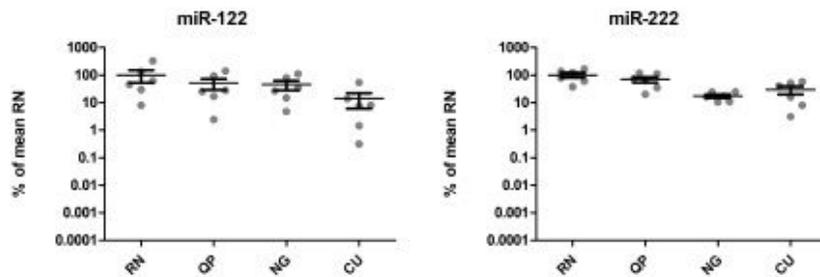
A serum



B urine



C perfusate



D bile

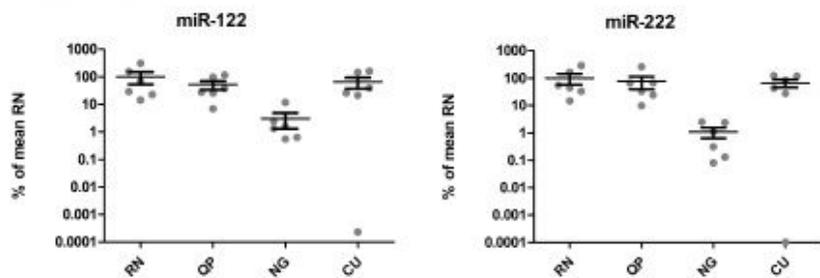


Figure 2

Quantification of endogenous miRNA recovery in four biofluids. A) Relative detection levels of endogenous human miRNAs miR-122, miR-222 and miR-21 in serum, B) miR-30a and miR-92e in urine, C) miR-122 and miR-222 in perfusate, and D) miR-122 and miR-222 in bile. The mean of the levels with the RN isolation method per individual miRNA per liquid were set at 100%. Bars represent the mean \pm SEM of

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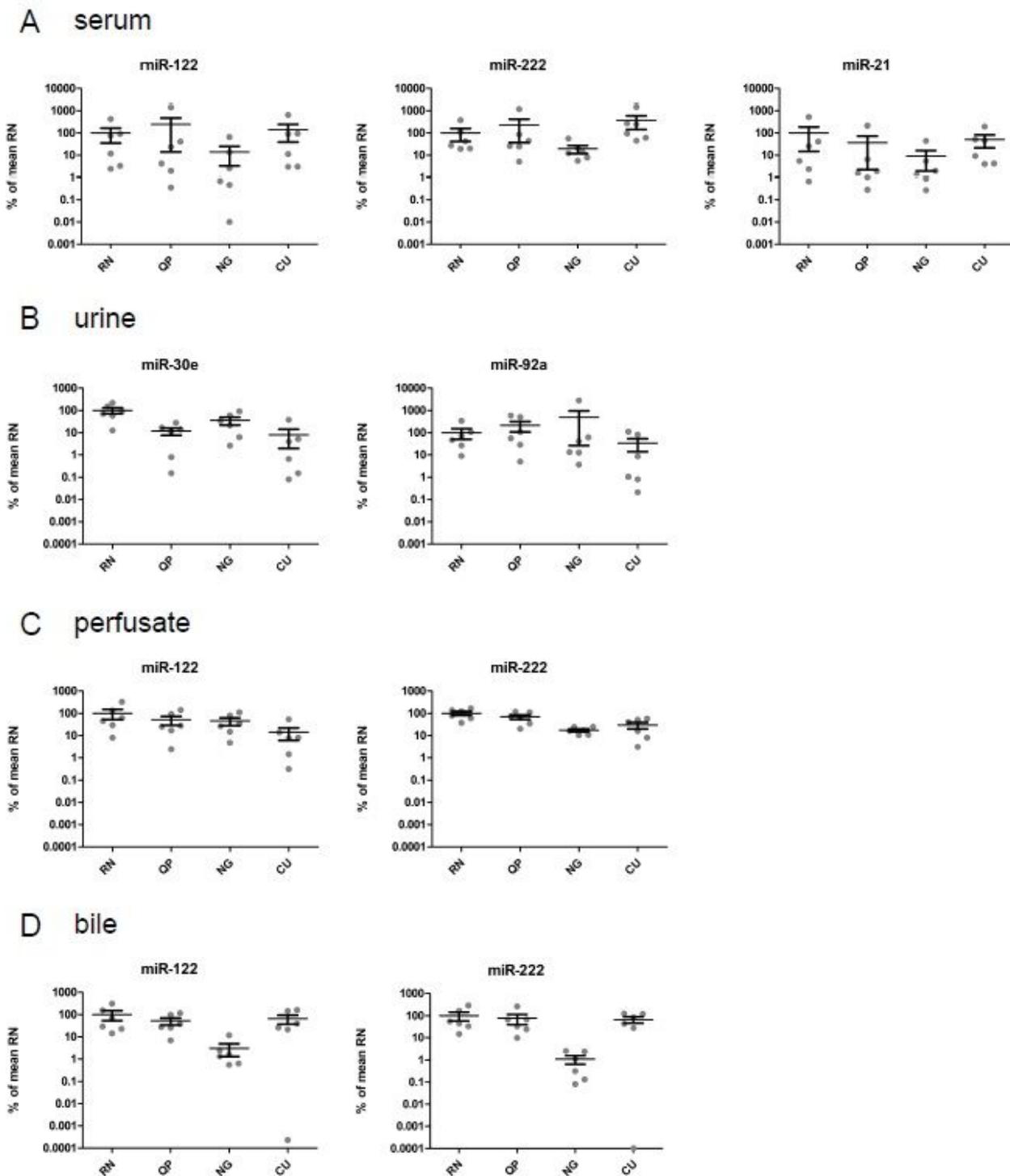


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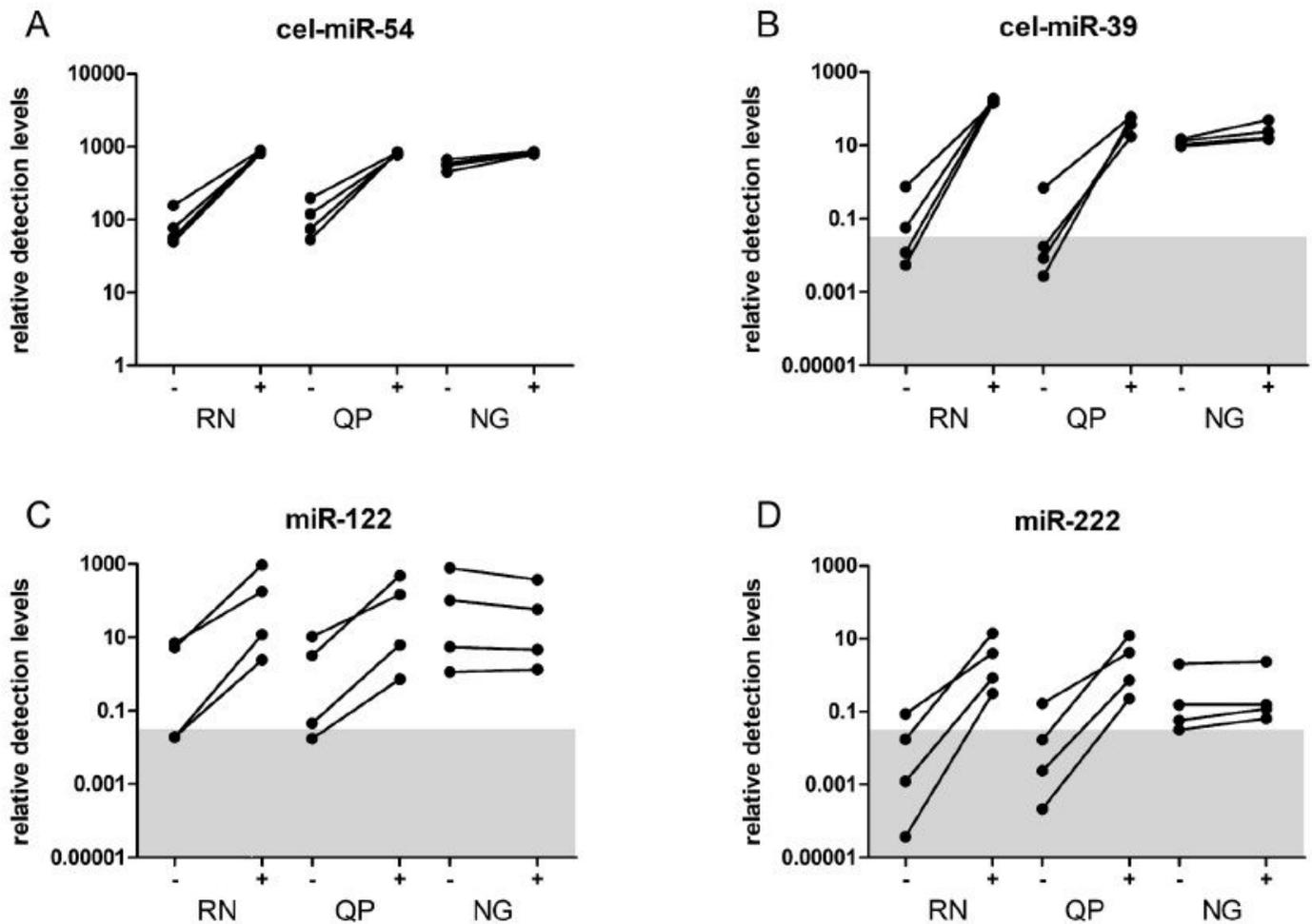


Figure 3

Heparinase I treatment improves quantification levels in biofluids with high heparin contamination. Total RNA from four different graft perfusate samples known to contain heparin were co-incubated with (+) or without (-) 6 IU heparinase I during cDNA synthesis. Results are shown for spike in miRNAs cel-miR-54 (A), cel-miR-39 (B) and endogenous miRNAs, miR-122 (C) and miR-222 (D) for three different RNA isolated methods, RN; Qiagen miRNeasy kit, QP; Qiazol in combination with the dr. GenTLE precipitation carrier, NG; NORGEN Total RNA isolation kit. Notably the NG method is the least sensitive for heparin co-purification and showed the smallest differences in detection levels between with or without heparinase I.

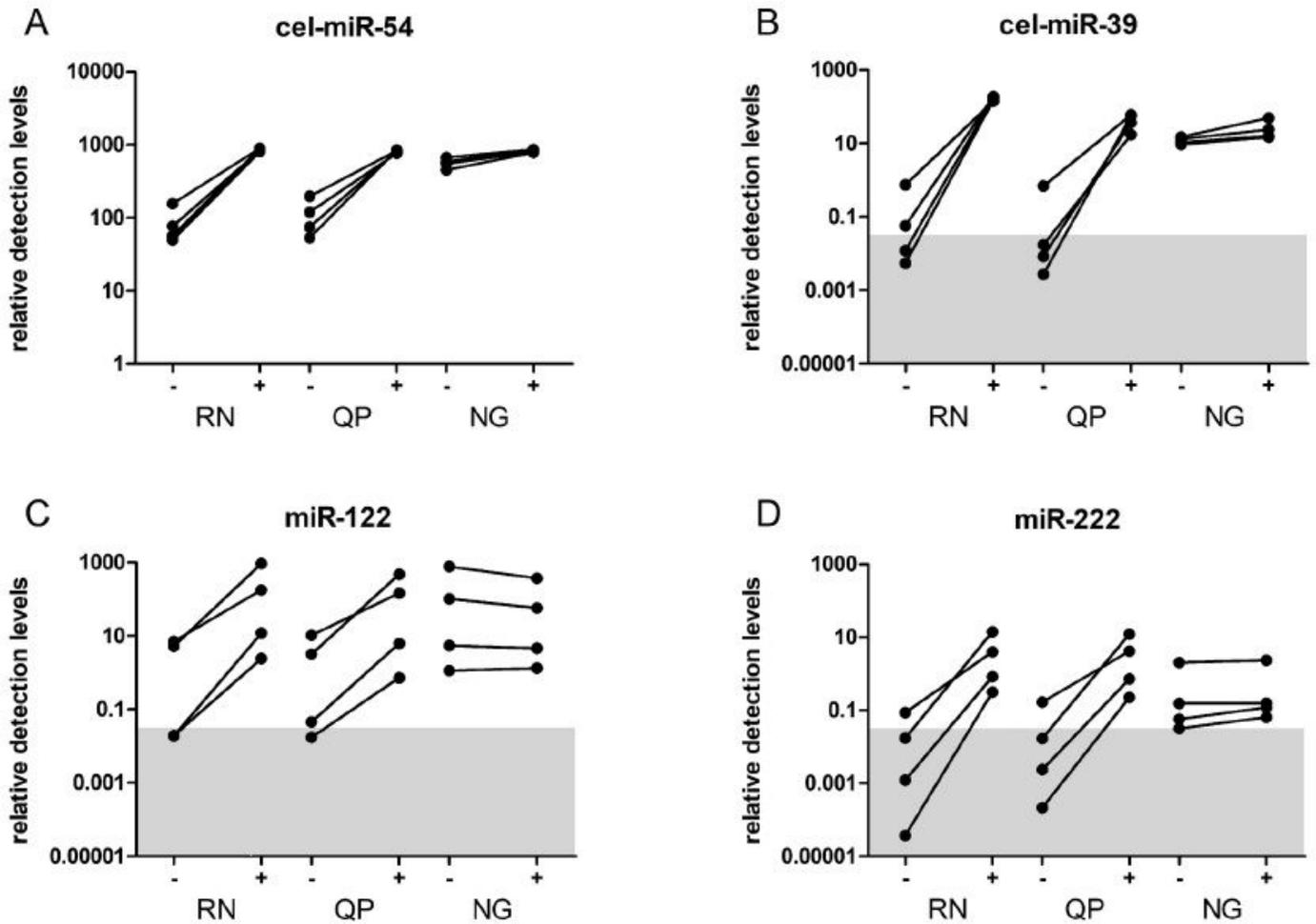


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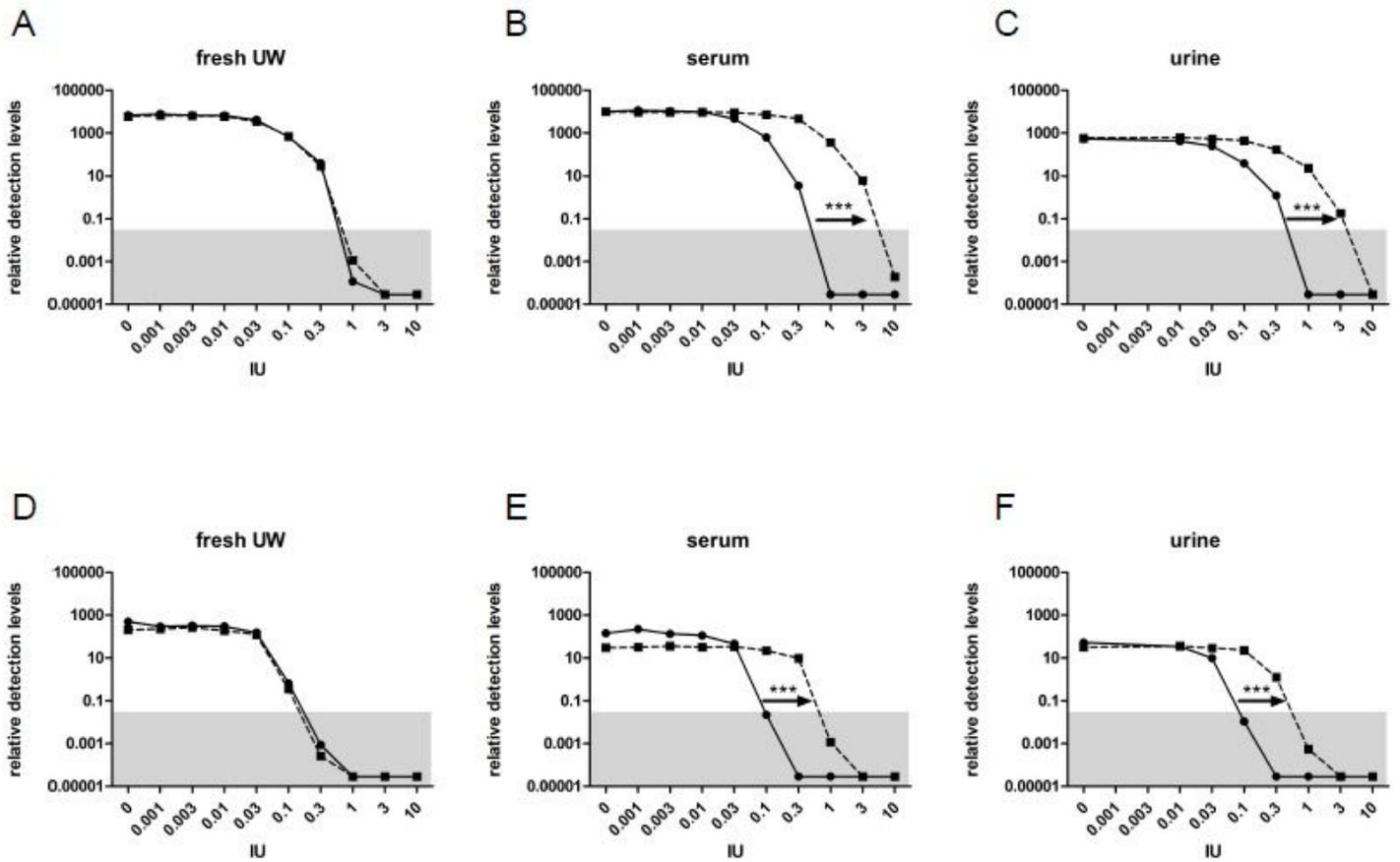


Figure 4

Differences in heparin sensitivity between isolation methods is only observed in body fluids. Biofluids were spiked with increasing amounts of unfractionated heparin. Ten μ l of a half-log dilution series containing the indicated IU of heparin were added to the samples prior to RNA isolation. Cel-miR-39 for loss during work-up (A, B, C) and cel-miR-54 as indicator of co-purified RT-qPCR inhibiting compounds (D, E, F) were determined in RNA from preservation fluid (fresh UW) (A, D), or from the body fluids serum (B, E), and urine (C, F), using method RN (miRNEasy kit, black circles/solid lines) and method NG (Norgen kit, black squares/dotted lines). Heparin amounts are shown on the x-axis. The area of unreliable detection is indicated in grey. In serum and urine, the inhibitory effect of heparin is approximately ten times higher with the RN method compared to NG method as indicated by arrows. No difference was observed for both spiked-in cel-miR-39 and cel-miR-54 isolated from fresh UW using method NG or RN. ***: $p < 0.001$, nonlinear regression (curve fit) comparison for dose-response data.

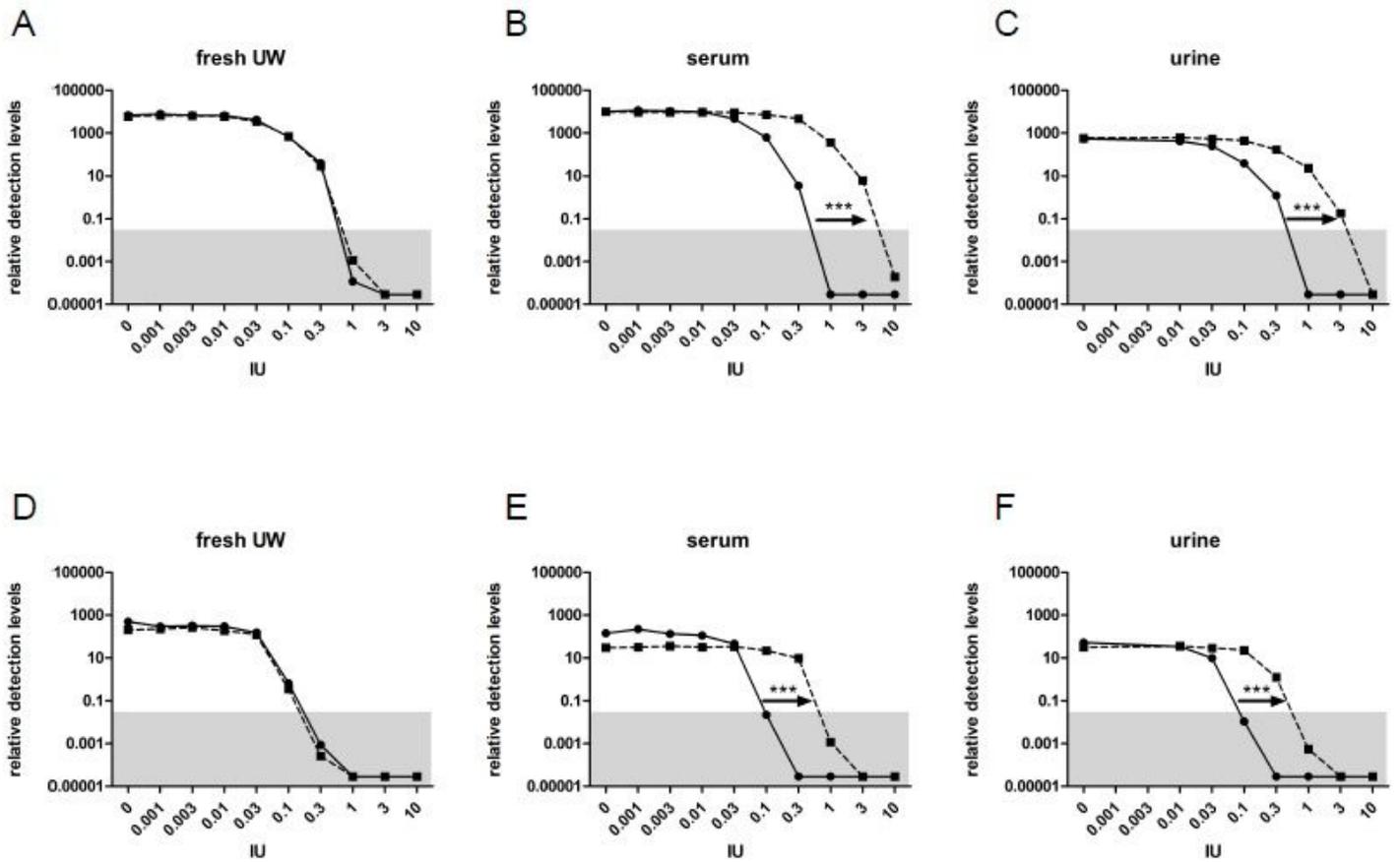


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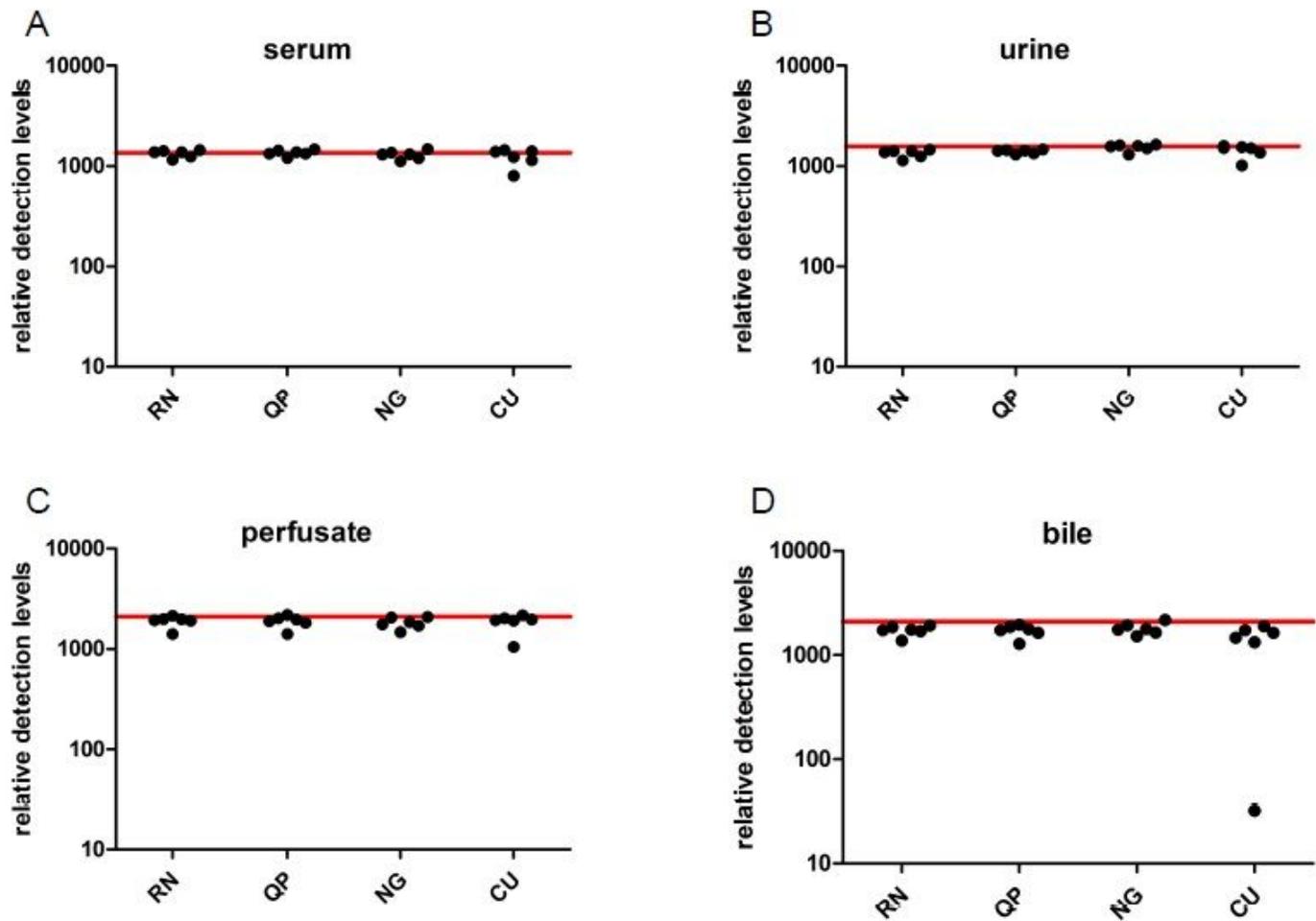


Figure 5

Relative detection levels of spiked-in cel-miR-54 in serum ,urine, perfusate and bile. Levels of cel-miR-54, spiked during cDNA synthesis, are determined in serum (A), urine (B), perfusate (C), and bile (D) from six liver patients and their donor organs. All samples were treated with Heparinase I and no statistical significant differences were observed between groups. The red line indicates the relative detection levels of the reference sample (cel-miR-54 spiked in RNA-free water). RN; Qiagen miRNeasy kit, QP; Qiazol in combination with the dr. GentLE precipitation carrier, NG; NORGEN Total RNA isolation kit, and CU; miRCURY RNA isolation kit – biofluids.

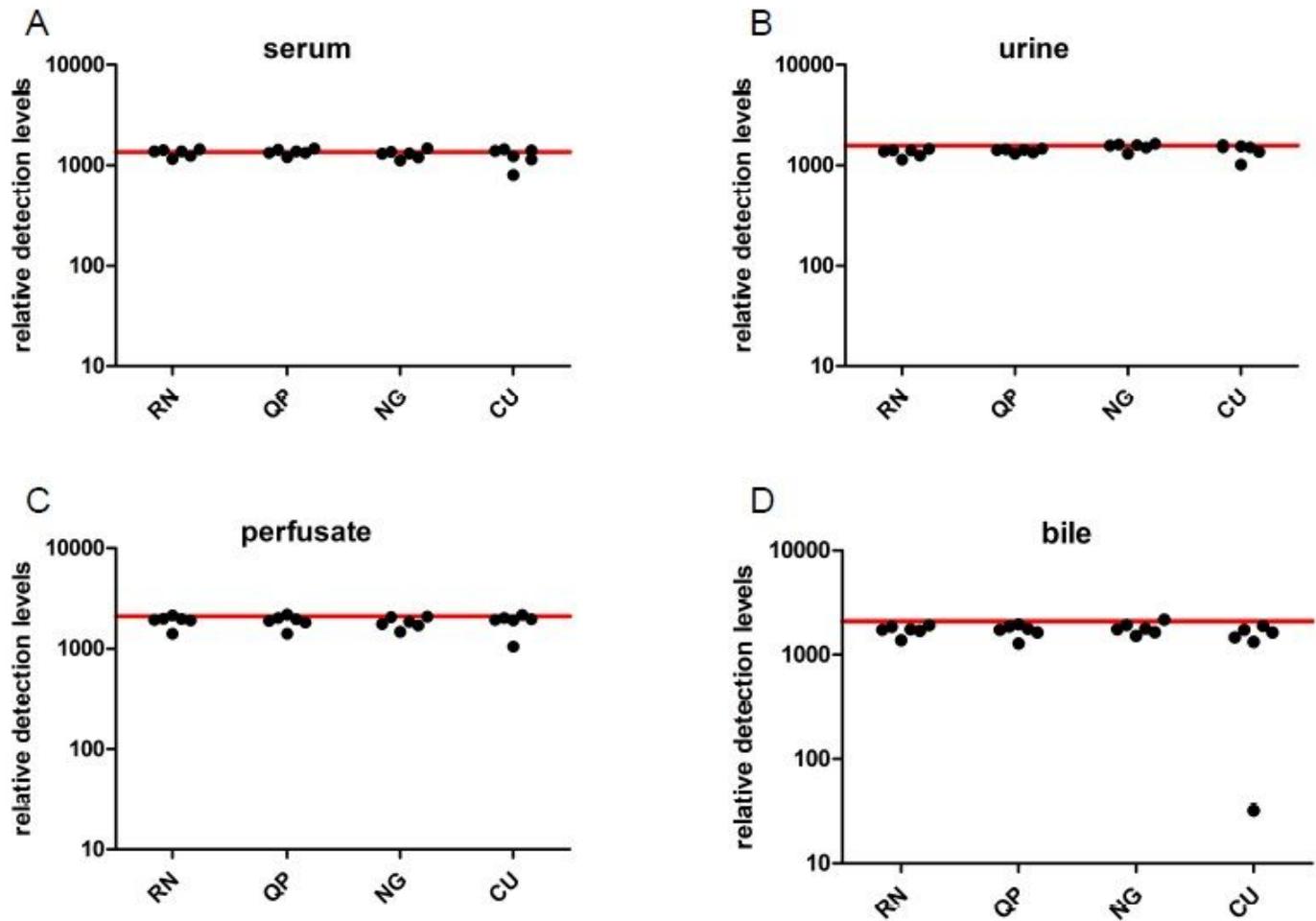


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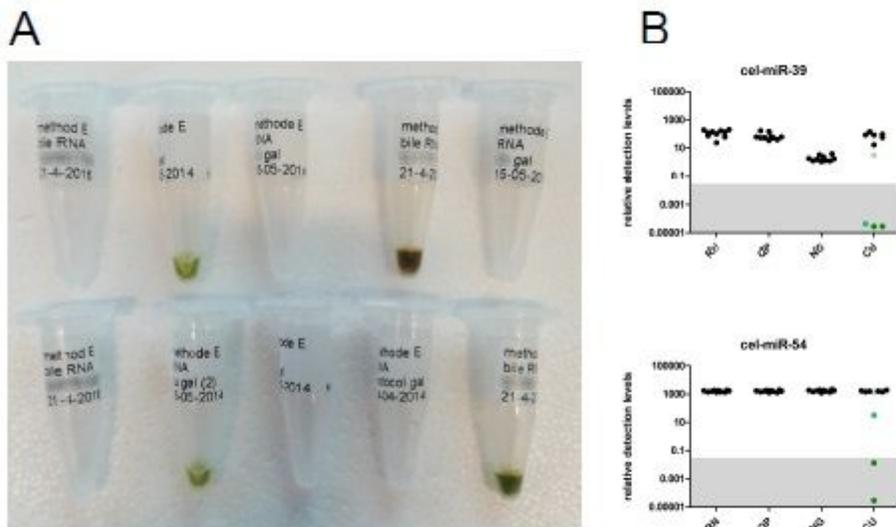


Figure 6

Colorimetric effects on RT-qPCR results in bile. (A) Visual observation of total RNA isolated from ten bile samples using the miRCURY RNA isolation kit – biofluids showed four samples had greenish discoloration. RNA samples isolated by other methods did not show any coloration (not shown) (B) The coloration of RNA samples correlated with low detection levels of cel-miR-39 (upper panel) and cel-miR-54 (lower panel). Black circles represent non-colored samples, green circles represent the greenish discolored samples. The unreliable detection area is indicated in grey. RN; Qiagen miRNeasy kit, QP; Qiazol in combination with dr. GentLE precipitation carrier, NG; NORGEN Total RNA isolation kit, CU; miRCURY RNA isolation kit - biofluids.

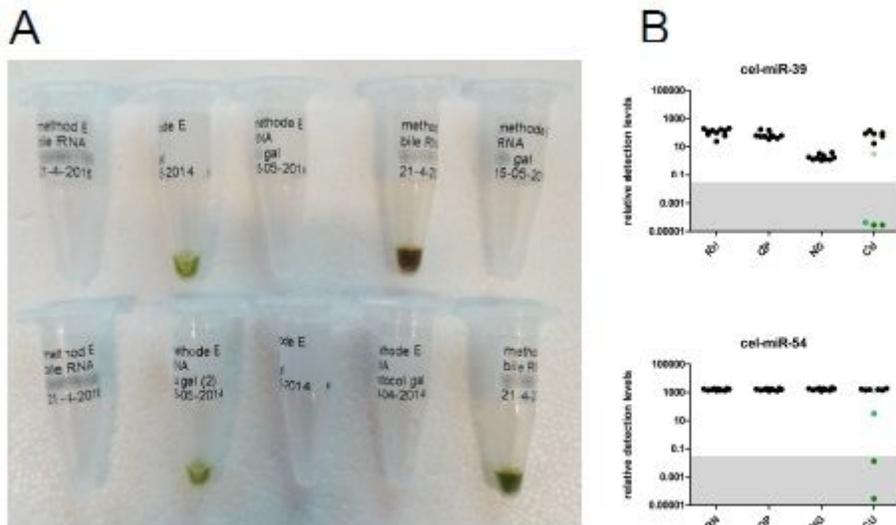


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