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Research Article

Optimizing microRNA quantification in serum samples

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Abstract

MicroRNAs constitute a group of small non-coding RNAs that negatively regulate gene expression. Aside from their contribution to biological and pathological pathways, altered expression of microRNAs have been reported in bio-fluid samples, such as serum etc. To employ serum's microRNAs as potential biomarkers, it is crucial to develop an efficient method for microRNA quantification, avoiding pre-analytical and analytical variations which could affect the accuracy of data analysis. Here, we optimized a real-time PCR quantification procedure for microRNA detection in serum samples. Serum total RNA was extracted using two different RNA isolation methods, one based on phenol-chloroform and the other based on silica column. To investigate a potential PCR inhibitory effect, different RNA amounts were subjected to reverse transcription. Moreover to assess the enzymatic efficiency, synthetic exogenous microRNAs was spiked into the mixture. To find a reliable internal control gene for normalizing the microRNA quantification, the amounts of 8 candidate non-coding RNAs including SNORD38B, SNORD49A, U6, 5S rRNA, miR-423-5p, miR-191, miR-16 and miR-103 were assessed on serum samples. Altogether, our data demonstrated that the silica-based method was more efficient for microRNA recovery. Furthermore, increasing the input volume of the extracted RNA would dramatically increase inhibitors' amounts which could end up in a larger Cq values. Therefore, the best input volume of RNA turned out to be 1.5 microliter/reaction. Among the 8 aforementioned internal controls, U6, SNORD38B and SNORD49A showed low levels of expression, and were undetectable in some samples. Amongst the others, 5s rRNA, had the biggest standard deviation which could significantly affect data analysis. MiR-103 with the least variations appeared to be the best normalizer gene.

Keywords: MicroRNA recovery, Real-time PCR quantification, Normalizer gene

Introduction

microRNAs (miRNAs) are a group of ~20 nucleotides non-coding RNAs that negatively regulate gene expression of their targets (Bartel, 2004). More than 60% of human protein-coding genes contain miRNA binding sites in their 3'UTR (Friedman et al., 2009). By direct binding to their mRNA targets, miRNAs play important roles in most cellular and developmental processes, and hence have been implicated in a large number of human diseases (Kloosterman and Plasterk, 2006). In parallel to their contribution to biological pathways, miRNA could be also easily detected in biofluid samples such as serum, urine, saliva (Ajit, 2012). The ease of tracing them in biofluid samples, along with their unique signature of expression in various diseases have made microRNAs a new generation of biomarkers (Etheridge et al., 2011). Although the exact function of secreted miRNAs is not fully understood (Chen et al., 2012), but recent findings indicate that cells can uptake secreted miRNAs as exogenous signals to fine-tune their interior regulatory network (Kosaka and Ochiya, 2011; Mittelbrunn and Sanchez-Madrid, 2012; Wang et al., 2010). It is also demonstrated that specific patterns of cell-free miRNAs are related to different pathological states (Brase et al., 2010; Fan

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et al., 2013; Taylor and Gercel-Taylor, 2008).

Due to growing propensity toward using cell free miRNAs as biomarkers it is critical to develop an efficient method for miRNAs evaluation to avoid pre analytical and analytical pitfalls while working with serum samples (Gilad et al., 2008; McDonald et al., 2011). One of the most important sources of pre-analytical variations is RNA extraction step (Eldh et al., 2012; McDonald et al., 2011) because of low concentration of RNA and abundance of proteins in serum samples, it is of great importance to choose a reliable RNA isolation method, otherwise loss of RNA load and co purification of PCR inhibitors could cause considerable deviation of the result and misleading data (Kroh et al., 2010; Mraz et al., 2009).

Although technical variations are inevitable, finding and applying a suitable internal control gene to normalize qPCR data would be an appropriate way to minimize analytical variations. Successful biomarker discovery projects are dependent on controlling for these sources of pre-analytical and analytical variations. To address aforementioned pitfalls, two conventional methods for serum RNAs recovery were compared. Moreover to minimize the effect of serum derived inhibitors the optimal starting volume of extracted RNA in cDNA synthesis step was evaluated. Furthermore, we showed the importance of a reliable normalizer gene for miRNA qPCR analysis.

Materials and methods

Blood collection and plasma preparation

Whole blood samples were collected from 20 healthy donors and directly drawn into serum separator tube (BD Vacutainer, Plymouth, UK). They were incubated for 1 hour at room temperature and then centrifuged for 15 min in 2500g. The clear supernatant was harvested in nuclease free tubes and stored in -80^{o}C until further investigations.

RNA extraction

Each serum samples was divided into two 200 µl portions. One was subjected to phenol-chloroform RNA extraction, using Trizol LS reagent (Invitrogen, USA). The other aliquot was used in silica column based RNA extraction, using miRNeasy mini kit (Qiagen, Germany). All Serum samples were completely thawed on ice and then, 20 fmol of synthetic Caenorhabditis elegans miRNA was spiked into the mixture. Extraction procedure was performed according to the manufacturer's instruction for both RNA isolation methods. In column based method, before adding Qiazol, MS2 RNA (Roche Applied Science) was added to each sample at the final concentration of 1 µg µl-1 to increase the yield of miRNA extraction. Adding this carrier would increase the final recovery of miRNA during extraction.

Reverse transcription

Synthetic spiked-in miRNA, miR-21 and 5s rRNA were reverse transcribed by commercially available primers (exiqon) and The miRCURY locked nucleic acid universal cDNA synthesis kit (exiqon, Denmark). In order to minimize PCR inhibitory effect of the serum derived RNA, several volume of RNA sample input were tested including 0.5, 2, 1, 1.5, 3 and 6 µl of RNA were reverse transcribed in a 10-µl reaction volume. Other reagents were applied according to the company's protocol. A non-template reaction, containing only exogenous synthetic spiked-in miRNA (a high quality RNA provided by exiqon), was considered as a control to determine the exact cDNA synthesis efficiency and also monitor PCR inhibitory effect.

cDNA for miRNA profiling, was synthesized using miRCURY locked nucleic acid universal cDNA synthesis kit following manufacturer's instruction.

Real-time PCR

Quantification of the synthetic spiked-in miRNA, miR-21 and 5s rRNA was done in 20-µl PCR reaction using the miRCURY SYBR Green kit and specific primer mix (exiqon, Denmark) through ABI 7500 Instrument (7500 Applied Biosystems, USA). All reactions were performed in duplicates. LinReg software was used to evaluate the PCR reactions efficiency.

Table 1. Statistical values of 8 candidates reference genes

Column1	min	max	std	average
SNORD49A	36.61	40	1.54	38.26
miR-423-5p	30.58	33.95	1.02	32.21
miR-103	27.64	30.2	0.74	28.73
miR-191	30.08	33.05	0.97	31.68
SNORD38B	35.76	40	1.90	37.51
miR-16	26.68	30.23	1.02	27.67
5s rRNA	20.07	34.15	3.21	26.63
u6 snRNA	32.98	40	2.13	36.13

We used the miRCURY LNA human miRNA Real-time PCR panel I and miRCURY SYBR Green kit for miRNA profiling experiments on serum RNA, with LightCycler instrument (Light Cycler 480, Roche Company, Germany). q-PCR data of 7 candidate reference genes including SNORD38B, SNORD49A, U6, miR-423-5p, miR-191, miR-16 and miR-103, provided in panel I and also of 5s rRNA (not provided in panel and was obtained using a separate reaction on samples), were use for finding an eligible internal control in serum (table 1).

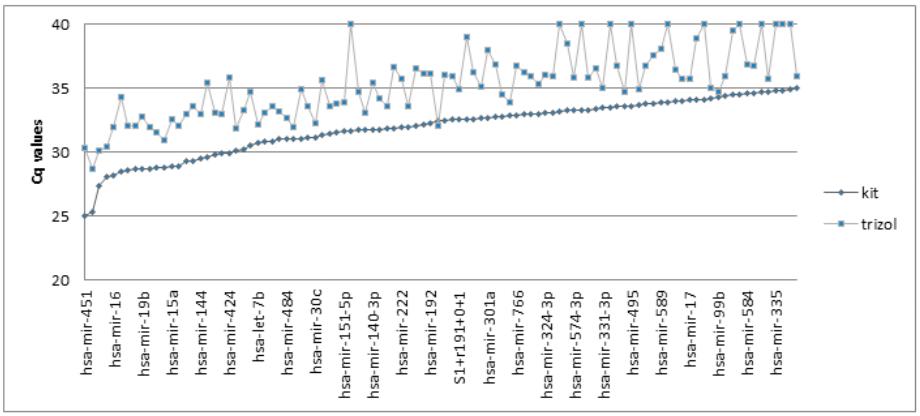

Fig. 1 Comparison of raw Cq values of 100 highly expressed microRNAs in two differentially extracted RNA samples groups

Statistical analysis

Standard deviations and student t test p values were calculated by GraphPad software and MS EXCEL. P values less than 0.01 considered to be significant.

Results

Pre-analytical optimization

Optimizing RNA recovery

One of the most challenging steps of miRNA quantification in serum samples is RNA extraction which is also considered as a source of pre-analytical variations. In addition to small RNAs loss during isolation step, potential PCR inhibitors could be introduced into the extracted RNA. After reviewing several related publications, we decided to test two frequently used isolation methods; first, extraction with Trizol® LS reagent which is based on phenolchloroform method and second isolation by miRNeasy mini kit that depends on silica filter column. The ability of two different RNA isolation methods for miRNAs recovery was investigated through qPCR. Our results showed superior recovery of miRNAs by the kit, with an average reduction in Ct values of 3.329 on all types of miRNAs, including those which are highly expressed (miR-21; miR-16) or those with very low amounts of endogenous transcripts (miR-192; miR423-5p). qPCR profiling on 20 normal samples showed that out of 380 miRNAs, included on panel I, only 68 miRNAs were detected in RNA samples, purified by Trizol (Cq <35 was included in analysis). Surprisingly this amount increased to 114 miRs in the kit purified RNAs. Comparison of raw Cq values of 100 highly expressed miRNAs in two differentially extracted RNA samples groups is shown in figure 1.

Evaluating the presence of inhibitors

Considering the low (minute) amount of secreted miRNA in serum samples, it is tempting to maximize the amount of input RNA used per reactions to compensate for such a low RNA yield. However, our data revealed that increasing the input amount of RNA could dramatically increase inhibitor amounts and end up in a larger Cq value. For example, miR-21's Cq value which was 28 for 1.5-µl RNA input reactions, increased to 40 when 3-µl RNA input was applied in the cDNA synthesis step.

Considering the Cq value of spiked-in in the non-template reaction as baseline we concluded that the volume of 1.5 µl RNA input has the minimum PCR inhibitory effect among five different examined volumes (fig 2). Moreover our analysis on two endogenous noncoding RNAs (5s rRNA and miR-21) was in accordance with spiked-in data (fig 3).

Analytical considerations

To find a reliable internal control gene, 8 non-coding RNA candidates available on panel I qPCR, were assessed on serum samples. Some of these genes including U6, SNORD38B and SNORD49A showed low level of expression which lead to their exclusion from further analysis. Statistical assessment on the remaining candidates indicate that miR-103 with the lowest standard deviation is the best normalizer gene. Surprisingly 5s rRNA, a frequently used internal control in miRNA studies, had the largest standard deviation. To have better insight on internal control effect on data analysis, we normalized miR-143 expression using miR-103

and 5s rRNA on 10 individuals (Fig. 3). As shown in the graph, normalizing Mir-143 expression using 5s rRNA gives a mean value of 8.7 while this value decreased to 4.1 when normalized by miR-103 (p value <0.0001). This large amount of variation could significantly affect the level of expression. Our other finding was in agreement with this statement that 5s rRNA cannot be a reliable internal control gene. As when we used an expired cDNA synthesis kit to evaluate robustness of 5s rRNA for evaluating the enzymatic efficiency, Cq values of 5s rRNA were near the mean range, while none of other targeted miRNAs were detected.

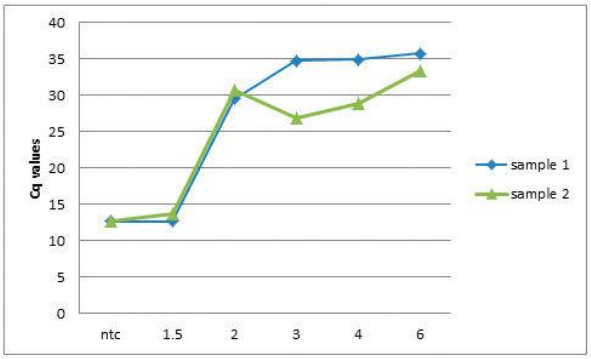

Fig. 2 Comparison of different volume of RNA sample input to find the volume with minimum inhibitory effect.

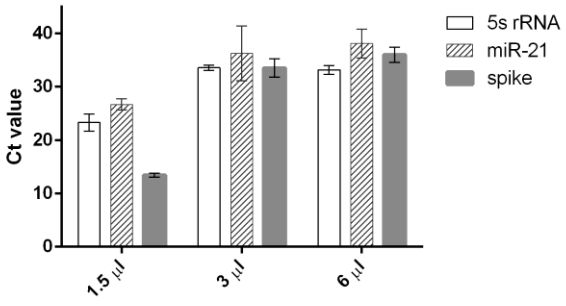

Figure 3. Comparison of Cq value of 3 targetes in 3 different volumes

Discussion

Recent studies introduce serum miRNAs as promising non-invasive biomarkers for varied biological and pathological conditions. Despite the accessibility and their ease of use, miRNAs quantification in bio-fluid are subjected to many technical challenges which should be addressed prior to starting the procedure.

The small amount of secreted RNA in serum samples make it critical to choose a robust RNA extraction method to ensure recovery of maximum RNA load. Herein, we demonstrate that silica column based RNA isolation methods are more efficient than the widely used phenol-chloroform methods. Moreover, organic particles are carried over during isolation step and high level of protein inhibitors in serum samples; make it crucial to evaluate the best starting input volume for minimizing inhibitory effects. Finally To achieve reliable and also reproducible qPCR data, non-biological variations, resulting from technical inconsistencies should be corrected using an appropriate reference gene, although finding a suitable reference gene for miRNA quantification in bio-fluid samples is a problematic step. Our findings revealed that non-miRNA reference genes like U6 and 5s rRNA could not be considered as powerful normalizers. Aside from large standard deviations they could not represent the actual efficacy of enzymatic reactions. This could be due to minute amount of miRNA compared to abundant RNA fraction of 5s rRNA in serum samples. Therefor the discrepancy in frequency of these two groups of non-coding RNAs (miRNA and rRNAs) leaves 5s rRNA out of potential miRNAs normalizer genes.

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