miRNA quantification and evaluation of suitable housekeeping genes in the major pre-clinical species

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INTRODUCTION

miRNAs (miRNAs) regulate more than 60% of cellular protein expression through inhibition of mRNA transcription and are thus of great interest in both therapeutic and diagnostic areas. miRNAs are highly conserved across species and circulate throughout the body in association with proteins, exosomes and vesicles. Numerous studies have demonstrated that miRNAs are highly sensitive and translatable biomarkers of drug-induced injury (1). As the number of novel miRNA biomarkers increases, there is a growing need for standardized and reproducible, extraction and quantification methods that are both sensitive and specific to the biological conditions. RT-qPCR has gained acceptance as a robust and reliable transcriptomic method to profile small changes in miRNA levels. For interpretation of results and to allow comparison between studies, the choice of a suitable reference miRNA, a so-called housekeeping gene, to use as an internal control is a crucial factor. To date, there has been no systematic evaluation of RT-qPCR reference genes for the study of miRNA expression in whole blood from different pre-clinical species. In this study, we validated the whole blood expression of two housekeeping miRNAs, miR-16 and miR-103, involved in cell differentiation and cell cycle, using two RT-qPCR kits: miRCURY LNA (Exiqon) and miScript (Qiagen) (2). After comparison of the sensitivity and PCR efficiency of the kits, the expression of these miRNAs was assessed in whole blood of male and female animals from five routinely used pre-clinical species: Sprague-Dawley rat, Swiss CD-1 mouse, Gottingen minipig, beagle dog, and cynomolgus monkey.

METHODS

Collection of blood from three male and female mice (100 µL), rats, minipigs, dogs and cynomolgus monkeys (500 µL). miRNAs were extracted using the RNeasy MiniElute Cleanup kit (Qiagen). Then miRNAs were reverse transcribed into cDNA using either mercury LNA® Universal RT miRNA PCR (Exiqon) or miScript System (Qiagen) kits. Exiqon method: 50 ng of miRNA was reverse transcribed using the Exiqon miRNA amplification kit and SYBR Green Master mix. Qiagen method: 20 ng of miRNA was reverse transcribed using the miScript Reverse Transcription kit with cDNA synthesis buffer. PCR efficiency of miR-16 and miR-103 was substantially better with the Exiqon kit (Figure 1). After 10-fold dilution, 5 µL of cDNA was used for PCR amplification using the ExiLENT SYBR Green Master mix. For both methods, primer sequences were as follows:

- miR-103: AGCACCAUGUUAGCGCCGCUAGGC
- miR-16: UACGCGACGCUAAUAUUUGCGC

In the presence of the target sequence, PCR amplification yields a fluorescent signal that rises above the baseline threshold before the final PCR cycle (cycle 40). PCR raw data are then defined as Cycle threshold (CT), i.e. the PCR cycle number for which the signal rises above the threshold. All sample data were analyzed in duplicate using Excel. RT-qPCR was performed in association with the Minimum Information for Publication of Quantitative Real-Time Experiments (MIQE) guidelines (3). An overview of the PCR systems is shown in Figure 1. Exiqon uses miRNA threshold. All sample data were analyzed in duplicate using Excel. RT-qPCR was performed in association with the Minimum Information for Publication of Quantitative Real-Time Experiments (MIQE) guidelines (3). An overview of the PCR systems is shown in Figure 3.

RESULTS

Comparison between Exiqon and Qiagen PCR kits

A calibration curve was prepared using a serial dilution of extracted miRNA in order to assess the PCR efficiency. PCR efficiency was calculated using the following formula: efficiency (%) = (10-1/slope-1) x100. PCR efficiency of miR-16 and miR-103 was substantially better with the Exiqon kit (Figure 2). Under optimized conditions (20 ng of input miRNA for Exiqon and 50 ng for Qiagen), for both miR-16 and miR-103, there was no difference between males and females (maximum difference of 0.7 Ct). As a delta Ct of 0.7 is the acceptance criterion for similar duplicates, this result indicates that males and females returned similar amounts of miR-16 and miR-103, which is in agreement with previous studies (4-8). With 5x10^-6 and 5x10^-5 compared to 2x10^-7 and 2x10^-6 ng miRNA, the limits of detection for miR-16 and miR-103 were at least two Ct lower with the Exiqon kit (Figure 2). In addition the Exiqon kit produced earlier Ct values for both miRNA tested. Indeed miR-16 and miR-103 Ct values with the Exiqon kit were at least two Ct lower than with the Qiagen kit, meaning that this method was 8-fold more sensitive for detection of miR-16 and miR-103.

Comparison between species

Generally, for both miRNAs, the Ct values were very similar in all the blood samples from the same species. The lowest differences (< 1 Ct) were observed between the six dog blood samples and the largest differences were observed between the six rat, mouse and minipig blood samples. In addition, lower Ct deviations were observed using the Exiqon kit compared to the Qiagen kit (Figure 3).

CONCLUSION

The relative quantification of gene expression by qPCR has become one of the major methods for studying miRNA expression in blood, but the value of this expression as biomarker investigations is strongly dependent on the selection of suitable reference genes. By extracting miRNA from blood and using a suitable quantification method such as that proposed by Qiu et al. (9), we were able to detect as little as 50 ng miRNA per 500 µL blood. This detection limit permits us to see small but important expression profile changes. These data demonstrate the suitability of miR-16 and miR-103 as reference (housekeeping) miRNA in whole blood using the Exiqon kit in the principal pre-clinical animal models.

REFERENCES