Notes & Tips

Feasibility of circulating miRNA microarray analysis from archival plasma samples

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A B S T R A C T

MicroRNAs have been found to be deregulated in several diseases and, due to their high stability in body fluids, represent promising noninvasively detectable biomarkers. However, numerous technical variables can affect accurate measurement of circulating miRNAs. Using a microarray-based method we assessed the: (i) adequate intra- and inter-array reproducibility of miRNA profiling; (ii) feasibility of using archival plasma samples stored for an extended period of time and available in limited amounts; (iii) good correlation between different batches; and (iv) time-dependent increase of background signals close to the chip expiration date.

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MicroRNAs (miRNAs) represent a class of small noncoding RNAs that regulate the expression of specific target messenger RNAs [1]. Aberrant miRNA expression has been reported in several diseases such as cancer, cardiovascular, metabolic, autoimmune, and neurological disorders [2]. A large body of work has highlighted the potential of miRNA signatures as biomarkers for diagnosis, prognosis, and treatment of numerous pathological conditions taking advantage of the extreme stability of miRNA in various types of clinical samples. Moreover, the discovery that miRNAs are also present in human body fluids (including blood, serum, and plasma) [3] in a stable form protected from endogenous RNAses has sparked interest in their use as potential biomarkers [4–6] and raised the possibility of using blood-based miRNA tests to complement clinical management through relatively noninvasive approaches.

Even though circulating miRNA profiling holds promise, there still are numerous preanalytical and analytical challenges in technical variables that can affect accurate analysis of circulating miR-

NAs in clinical practice. With regard to preanalytical variables, we have recently demonstrated that adequately treated, heparin-collected archival plasma samples are suitable for miRNA expression analysis, without affecting the performance of miRNA detection [7]. Regarding analytical aspects, the low abundance of miRNA in body fluids can potentially limit their reproducible detection and it is still unclear whether archival samples stored for an extended period of time are suitable for this type of analysis. We addressed these crucial issues using a microarray platform because it allows reaching a higher throughput than PCR-based assays and is expected to be advantageous in a discovery setting. In a cross-platform comparison experiment, we recently demonstrated that the Agilent miRNA microarray platform was the best system to measure miRNAs extracted from human tissues [8]; thus, we tested the performance of Agilent microarrays in measuring miRNA expression in archival plasma samples.

Plasma samples used in this study belong to the human plasma collection of the Fenretinide Breast Cancer Prevention Trial [9,10] and RNA was extracted from 350 μl (Materials and methods are detailed in the Supplementary material) [7]. According to recent reports by Kirschner et al. [11] and McDonald et al. [12], we observed that the low RNA yield in plasma hampered exact RNA quantification, and did not allow reliable absorbance at 260 nm measured with a NanoDrop spectrophotometer (data not shown). Moreover, Wang et al. recently found no correlation between RNA concentration and the number of circulating miRNAs de-

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tected, speculating that using similar amounts of RNA is not a useful method for quantifying miRNAs [13]. Consequently, we decided to perform chip hybridizations using an equal volume of each sample, also taking into account that the total amount of plasma/serum RNA could be affected by pathological conditions [11].

A total of 11 archived plasma samples collected between 1988 and 1999 were evaluated in this study. First, to assess intra- and inter-array reproducibility RNA extracted from two samples (S1 and S2, collected in 1999 and 1994, respectively) was hybridized in triplicate. To reduce the potential technical limitation represented by the low miRNA amount in plasma, the samples were also hybridized in triplicate after increasing the starting material volume from 2 to 2.5 µl, replacing optional spike-ins (i.e., *Drosophila* miRNAs). Although spike-ins could help in assessing the labeling and hybridization efficiencies, they could not be used in the normalization process of plasma miRNA expression (see Supplementary methods for details). All replicates of each sample were hybridized on the same chip. Median intra-array coefficients of variation were low in all replicates (median = 5.5%, range 4.9–7.6%) (Fig. 1A) and were in agreement with literature data on tissue samples [14], thus indicating a high precision of the technique in this setting. Moreover, despite the long-term storage of the plasma samples, the array results showed that more than 100 miRNAs were detected and correlations between technical replicates were higher than 0.99 even before normalization (Fig. 1B and C). The increase of the volume of labeled RNA enabled an increase in the

number of detected miRNAs: 12% and 13%, on average, in samples S1 and S2, respectively (Fig. 1B). The gain in miRNA detection was lower than the increase in the input volume (25%) but we can assume that a linear relationship between the input RNA and the number of detected miRNAs is not expected, as the number of annotated miRNA is limited and not all of them are likely to be significantly present in plasma samples.

To test the interbatch variability, which represents an important issue during biomarker development, as training and validation tests are usually carried out on different microarray batches, RNA obtained from 9 additional plasma samples (collected between 1988 and 1998) was processed and hybridized on two different batches of arrays. Correlation between replicates in the two batches, named A and B, was 0.89 (range 0.80–0.95), and the miRNA detection rates were similar ($R = 0.91$) (Fig. 2A). A good agreement in detected and not detected individual miRNAs was observed between A and B hybridizations (Cohen's K coefficient median = 0.71, range = 0.61–0.77). As expected, the agreement was prominently observed for miRNAs with the highest signal levels, while for miRNA close to background intensities the detection was weakly reproducible (Fig. 2B). As a consequence, a filtering step in the processing of such data could be advantageous. For example, only miRNAs detected in at least 50% of samples and/or those with an average expression higher than an empirically established cutoff could be selected for further analysis. Moreover, the results revealed that good detection rates can also be obtained
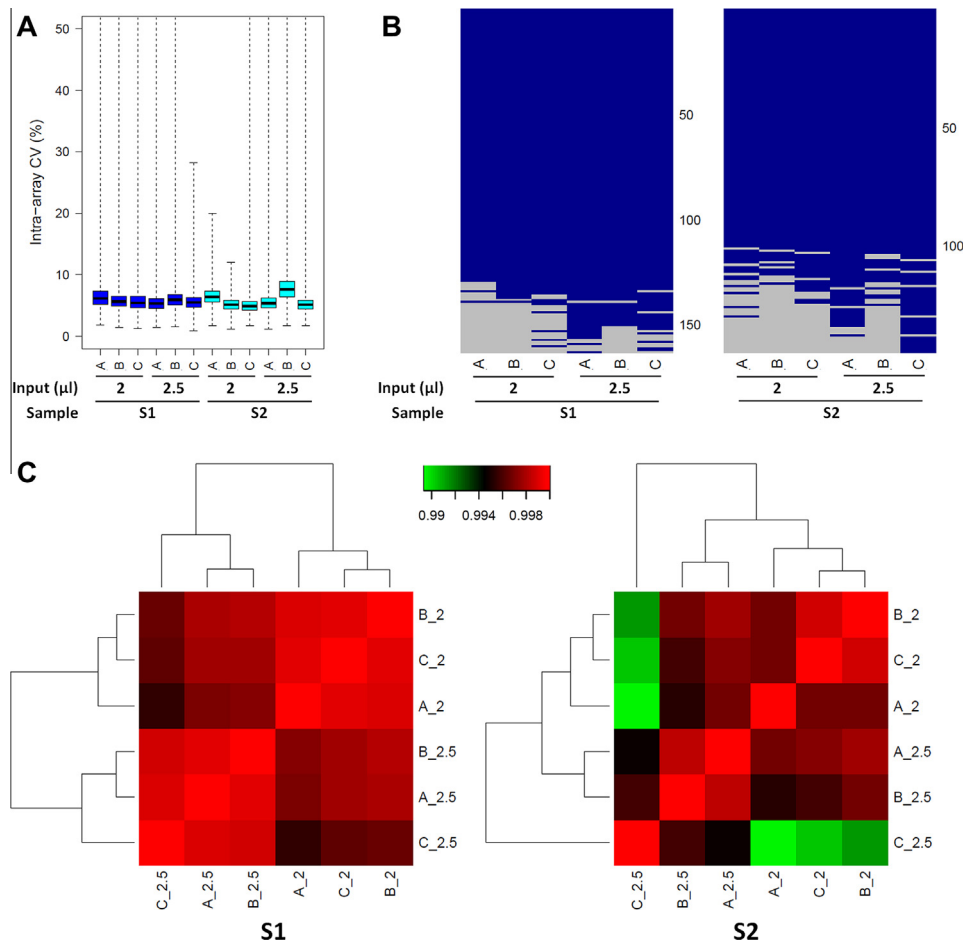

Fig.1. Intra- and inter-array reproducibility. Hybridizations of two samples (S1, S2) performed after labeling of 2 or 2.5 µl of total RNA. (A) Intra-array coefficients of variation (CV) across replicated spots on each array were computed for the unprocessed intensities of 3101 probes targeting human miRNAs. The *y* axis was truncated at 50% for clarity of presentation, excluding some extreme outliers. (B) miRNAs detected in at least one replicate of samples S1 and S2. Detected miRNAs are in blue and undetected in gray. (C) Pearson correlation among technical replicates for samples S1 and S2.
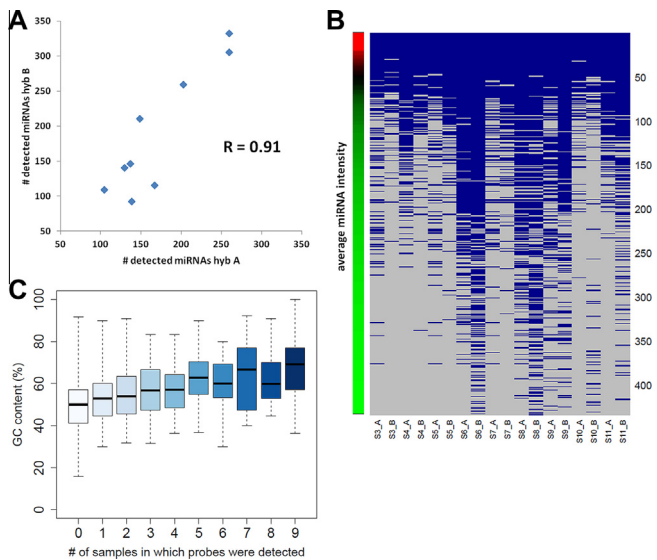
Fig.2. Effect of array batch and GC content on miRNA detection. (A) Nine samples were hybridized using different batches (A and B). Pairwise correlations for the number of detected miRNAs were calculated and *R* value is reported. (B) miRNAs detected in at least one replicate of the 9 samples hybridized as in (A) were plotted according to average miRNA intensity levels. Detected miRNAs are in blue and undetected in gray. (C) Box plot of the GC content as a function of the number of samples in which each probe is detected (ranging from 0, for probes detected in any sample, to 9, for probes detected in all samples). A significant difference in GC content between never detected and always detected probes was found ($P = 3.7 \times 10^{-33}$).

from samples older than 20 years, supporting the possibility of using archival plasma samples for retrospective studies. In addition, we noted that the number of samples in which each probe was detected depends on its GC content: miRNAs detected in all 9 samples showed a median GC content greater than 60% (Fig. 2C). This could be attributed to technical issues related to the hybridization process, as we and others described for tissue miRNA profiling [8]. However, we cannot exclude that miRNA stability in circulation might be affected by their GC content; further investigations are needed in this regard.

Noteworthy, signal levels from plasma samples were far lower than those from tissue/cell samples; thus, each source of technical variability resulting in an increment of background levels could negatively impact measurement of circulating miRNAs expression. Since a potential increase in background signals could be due to the length of array storage (the used chips had a lifetime of 6 months), we tested whether the use of slides close to their expiration date could affect the hybridization results. To this aim, two samples (S3 and S4) were rehybridized on an array of the same batch of the hybridization A (performed within the first month from the chip delivery), but just 1 week before the nominal expiration date (named hybridization C). We found a clear drop in the number of detected miRNAs in parallel with an increase in fluorescence and signal-to-noise ratio of negative controls (Fig. S1).

In conclusion, our data obtained using the Agilent's SurePrint G3 Human v16 miRNA microarrays showed: (1) a satisfactory intra- and inter-array reproducibility; (2) an increased number of detected miRNAs by increasing the input material; (3) a good correlation between different batches. However, we cannot rule out the possibility that a batch effect could potentially affect the evaluation of circulating miRNA biomarkers in large clinical studies. Moreover, the use of chips as far as possible from their expiration date seems to be critical. This study shows the feasibility of using archival plasma samples for miRNA profiling with high-

throughput microarray-based strategies, even if they have been stored for an extended period of time and the amount of available samples is limited.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.03.002.

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