

# MicroRNA expression analysis by LNA™-enhanced real-time PCR

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## Summary

MicroRNAs (miRNAs) comprise a family of highly conserved small non-coding RNAs (~22 nt). As regulators of post-transcriptional gene expression, miRNAs play an essential role in many parts of development, differentiation, and physiological processes. It is now established that altered miRNA expression profiles are associated with a number of different diseases including heart disease, neurological disorders and human cancers. This suggests the use of miRNAs as a novel class of biologically important biomarkers for disease diagnosis and prognosis. The study of expression and functional effects of miRNAs is complicated by their small size and limited availability

of sample. We have developed a real-time PCR method for quantification of miRNAs. The miRCURY LNA™ microRNA PCR system offers the possibility for highly sensitive and specific quantification of miRNA expression levels from total RNA. (for details please see accompanying poster).

A panel of endogenous control primer sets has been developed for accurate and sensitive quantification of small nucleolar RNAs, U6 snRNA and 5S rRNA. These can be used as normalizers, allowing comparison of miRNA expression levels. The endogenous controls show high levels of expression stability in a range of samples. However, for each set of samples, a different control or set of controls will be the most stable and

therefore the most optimal normalizer. The endogenous controls must always be empirically validated for each study. Even very small changes in miRNA expression levels, e.g. in comparing different disease stages, might be biologically significant. Reliable normalization is therefore critical when analyzing differences in miRNA expression. We show an example of how the use of a poor normalizer can lead to incorrect conclusions when studying differential miRNA expression. Finally, we demonstrate the excellent correlation between miRNA expression analysis using miRCURY LNA™ Arrays and the miRCURY LNA™ microRNA PCR system.

Figure 1: Schematic overview of the miRCURY LNA™ PCR System

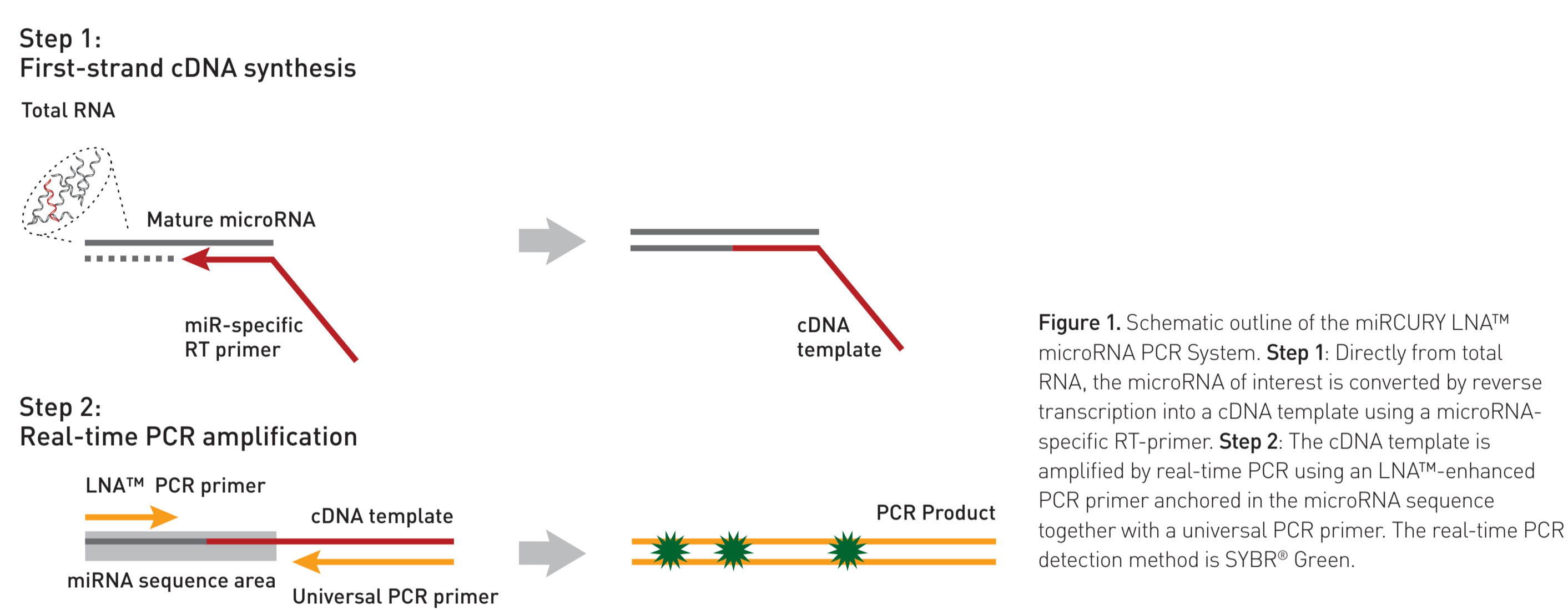


Figure 1. Schematic outline of the miRCURY LNA™ microRNA PCR System. **Step 1:** Directly from total RNA, the microRNA of interest is converted by reverse transcription into a cDNA template using a microRNA-specific RT-primer. **Step 2:** The cDNA template is amplified by real-time PCR using an LNA™-enhanced PCR primer anchored in the microRNA sequence together with a universal PCR primer. The real-time PCR detection method is SYBR® Green.

Figure 2: miRCURY LNA™ PCR Endogenous control primer sets

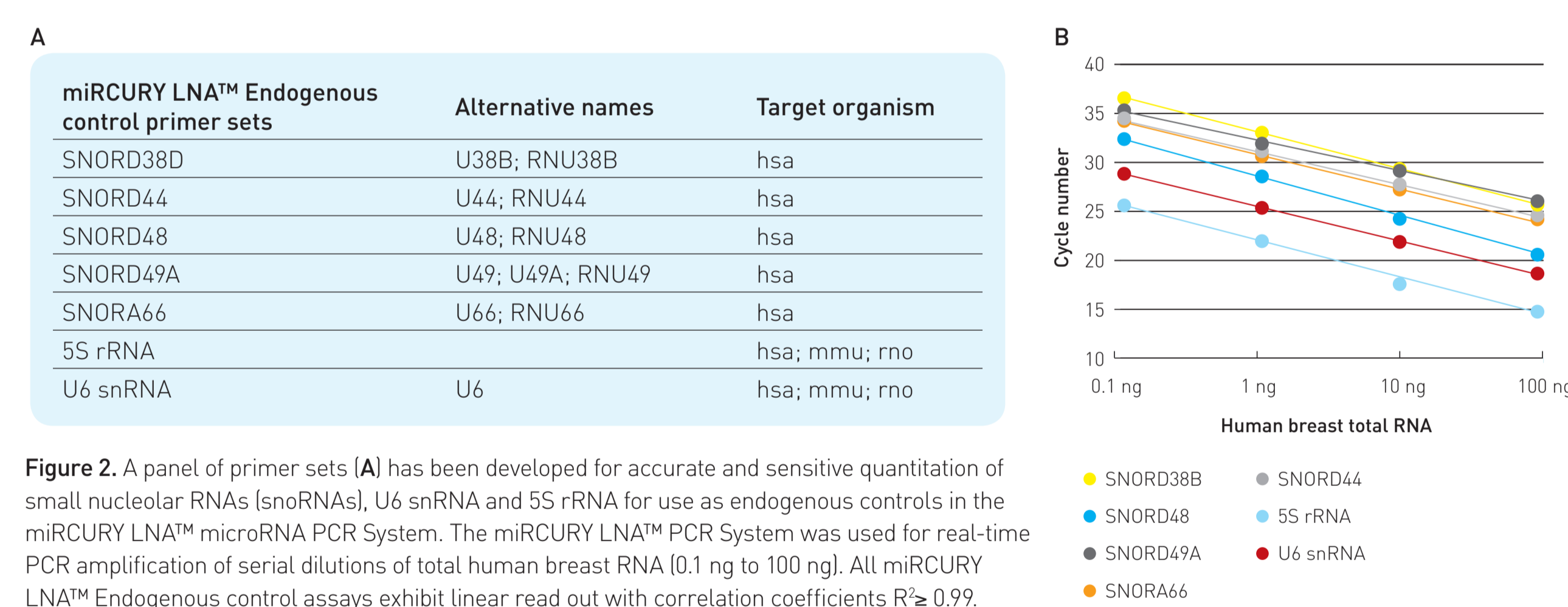


Figure 2. A panel of primer sets (A) has been developed for accurate and sensitive quantitation of small nucleolar RNAs (snoRNAs), U6 snRNA and 5S rRNA for use as endogenous controls in the miRCURY LNA™ microRNA PCR System. The miRCURY LNA™ PCR System was used for real-time PCR amplification of serial dilutions of total human breast RNA [0.1 ng to 100 ng]. All miRCURY LNA™ Endogenous control assays exhibit linear read out with correlation coefficients  $R^2 \geq 0.99$ .

Figure 3: Expression profile of Endogenous controls

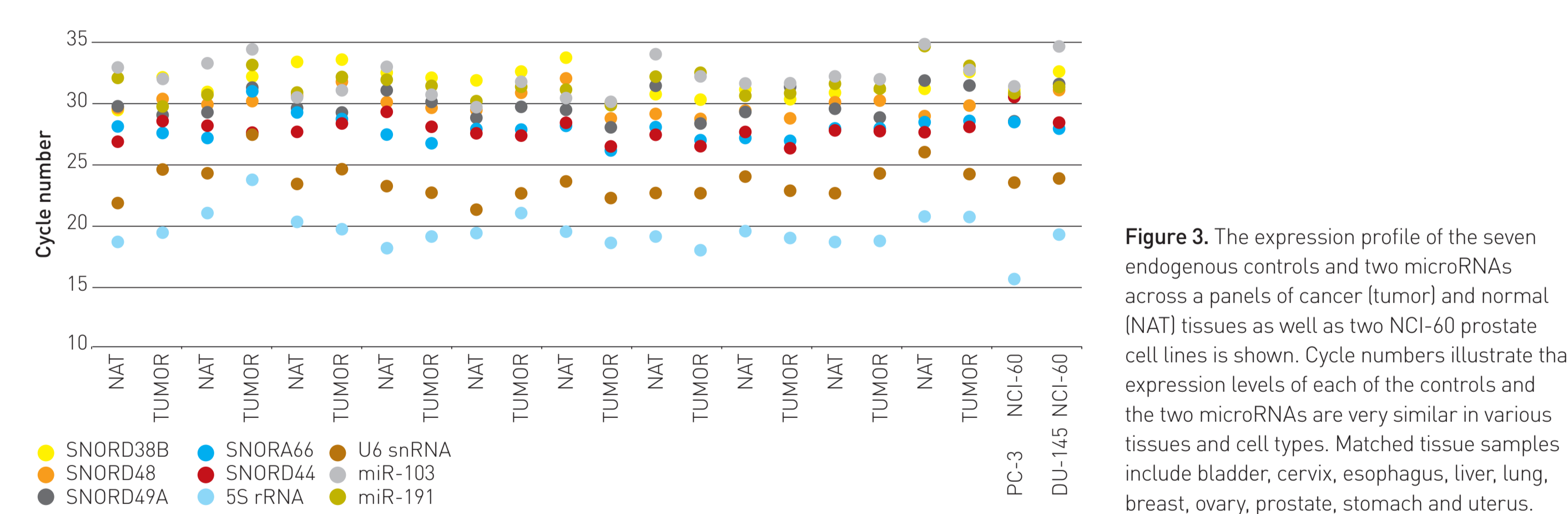


Figure 3. The expression profile of the seven endogenous controls and two microRNAs across a panels of cancer (tumor) and normal (NAT) tissues as well as two NCI-60 prostate cell lines is shown. Cycle numbers illustrate that expression levels of each of the controls and the two microRNAs are very similar in various tissues and cell types. Matched tissue samples include bladder, cervix, esophagus, liver, lung, breast, ovary, prostate, stomach and uterus.

Figure 4: Average expression stability of Endogenous controls in three different sample combinations

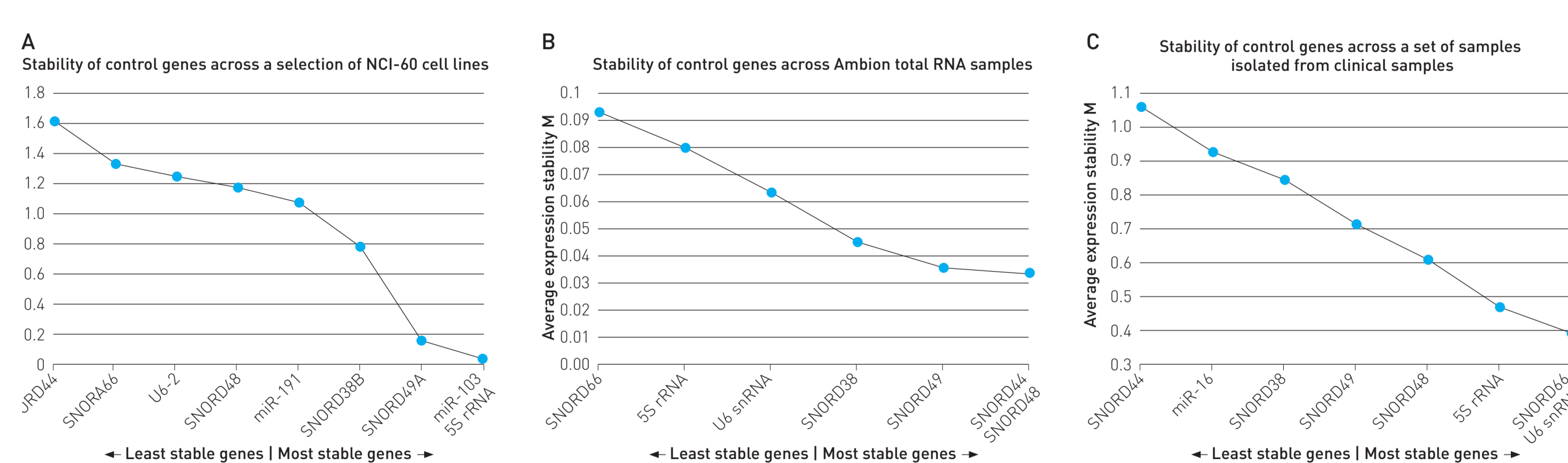


Figure 4. The geNorm algorithm was used to evaluate the expression stability of the endogenous controls in three different sets of total RNA samples from cell lines (A), tissues (B) and clinical samples (C). The average expression stability for each of the control genes is different depending on the type of sample used and in each set of samples a different pair of genes is designated as the most stable. These results illustrate the importance of screening and validating a number of endogenous controls for each microRNA real-time PCR study. Experimental details: For each type of sample, 10 ng of total RNA was used. For each endogenous control primer set and each sample, 2 cDNA synthesis reactions and 2 PCR reactions per cDNA was performed and the average cycle number used.

Figure 5: Real-time PCR normalization with miRCURY LNA™ Endogenous controls

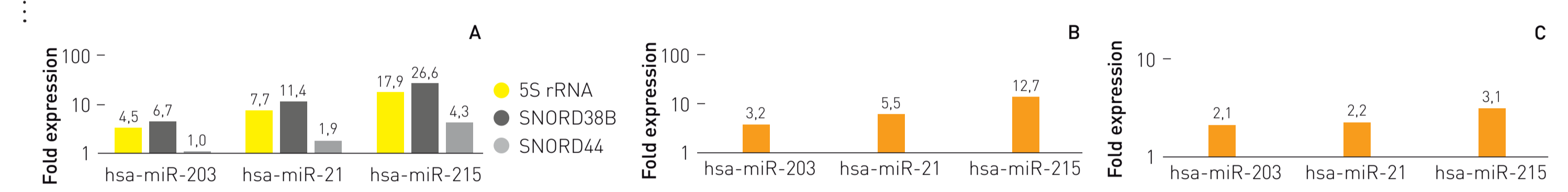


Figure 5. Use of a poor normalizer can lead to incorrect conclusions from microRNA expression studies. Three miRCURY LNA™ Endogenous controls 5S rRNA, SNORD38B and SNORD44 were applied for quantitation of hsa-miR-21, hsa-miR-203 and hsa-miR-215 in an example of matched tumor versus normal adjacent tissue (NAT) (A) Expression profile of hsa-miR-21, hsa-miR-203 and hsa-miR-215 from real-time PCR analysis upon single gene normalization. The figure shows fold regulation after normalization with each of the endogenous controls individually. (B) Expression profile of hsa-miR-21, hsa-miR-203 and hsa-miR-215 from real-time PCR analysis upon multiple gene normalization. Bars illustrate fold regulation after normalization with SNORD38B, SNORD44 and 5S rRNA in combination. (C) Normalized expression profile of hsa-miR-21, hsa-miR-203 and hsa-miR-215 from miRCURY LNA™ Array analysis.

Figure 5, experimental details: Real-time PCR was performed using the miRCURY LNA™ PCR System on 10 ng of total RNA. Fold regulation in tumor samples was calculated by setting the expression level to 1 in all NAT samples. For miRCURY LNA™ Array analysis the miRCURY LNA™ Array Labeling kit was used for labeling 1 µg of total RNA with Hy3™ (NAT) or Hy5™ (tumor tissue). Array data were normalized using global LOWESS.

Figure 6: Excellent correlation between data from miRCURY LNA™ Arrays and miRCURY LNA™ microRNA PCR System

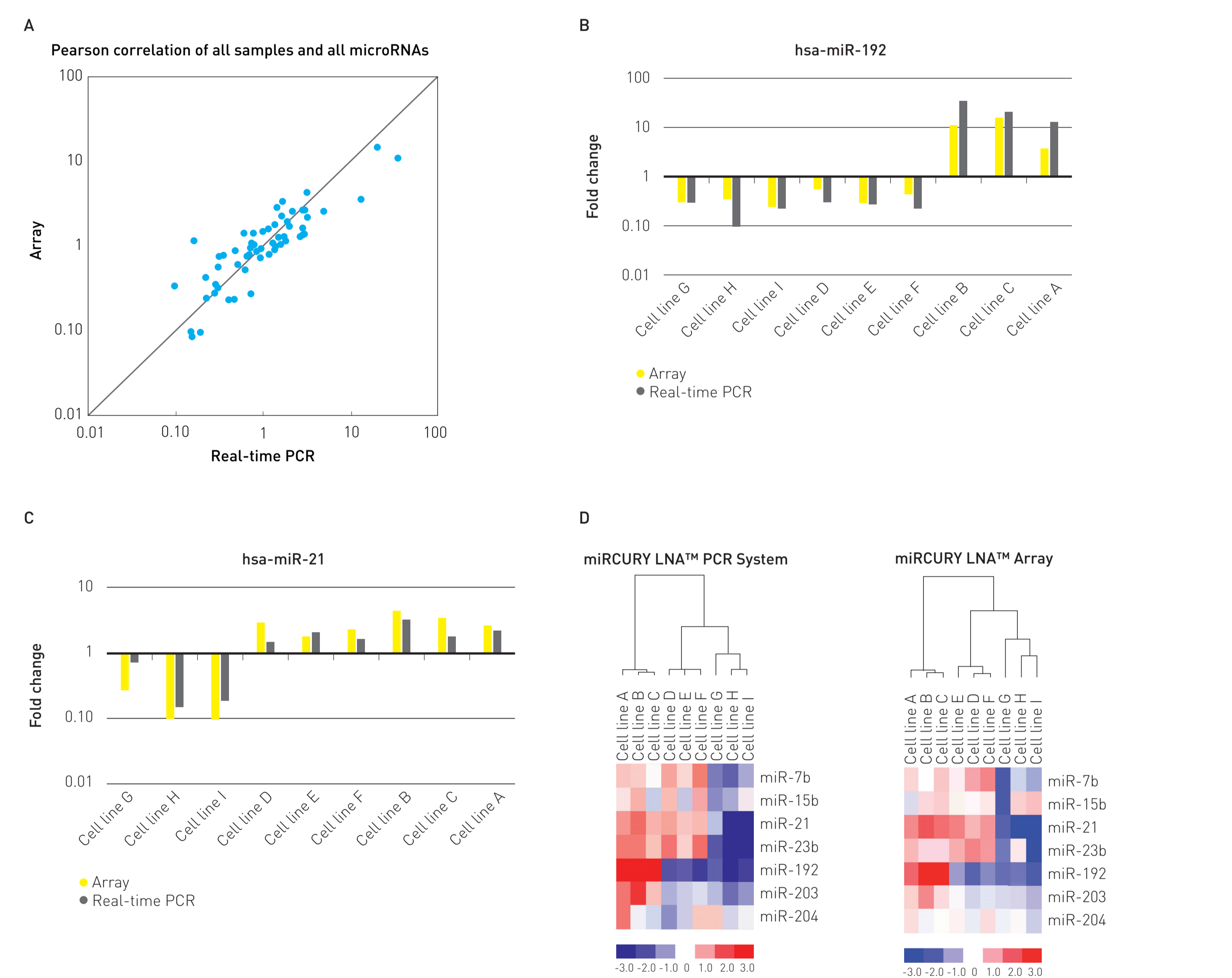


Figure 6. Nine cell lines were analyzed for differential microRNA expression using miRCURY LNA™ microRNA Arrays (see details below). Seven microRNAs were selected for real-time PCR analysis using the miRCURY LNA™ microRNA PCR System (see details below). The real-time PCR results were normalized using the geometric mean of five endogenous controls. There was excellent overall correlation between fold changes detected on the array and fold changes detected using real-time PCR (A). The direct comparison of array and real-time PCR results for individual microRNAs is exemplified by hsa-miR-21 (B) and hsa-miR-192 (C). Finally a two-way clustering of both array data and real-time PCR data generates very similar heat maps showing that the nine cell lines are clustered in the same way using miRCURY LNA™ microRNA Arrays or miRCURY LNA™ microRNA PCR System (D).

Figure 6, experimental details: Real-time PCR experimental details: 10ng total RNA from each cell line was used in two separate RT reactions. From each RT reaction, two real-time PCR reactions were performed and the median Cp values used. The five endogenous controls had geNorm average expression stability values between 0.35 and 0.65. The real-time PCR data were normalised with a normalisation factor generated using the geometric average of the expression of the Endogenous control genes. Fold changes ( $\Delta\Delta Ct$ ) for individual microRNAs were calculated by first converting Ct values to relative quantities [ $E^{-\Delta Ct}$ ] and finally log transformed. microRNA Array experimental details: 1g total RNA was labelled with Hy3 and hybridised against a reference sample labelled with Hy5. For each microRNA, the log median ratios (over 4 capture probes) were background corrected and normalised using LOWESS.