



Endogenous controls for the miRCURY LNA™ microRNA PCR System

Normalization of microRNA expression levels during real-time PCR analysis

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Importance of normalization

The miRCURY LNA™ microRNA PCR System offers the possibility for highly sensitive and specific quantitation of microRNA expression levels. Proper normalization is important for a correct analysis and interpretation of results from any real-time PCR gene expression experiment. Even very small changes in microRNA expression levels, e.g. in comparing different disease stages, might be biologically significant. Reliable normalization is therefore critical when analyzing differences in microRNA expression. Poor normalization can lead to incorrect conclusions regarding the magnitude and even direction of fold change when studying differential expression^{ref1}.

The purpose of normalization is to remove as much variation between samples as possible, which can mask important biological changes^{ref2}. Technical variation may arise from the differences in sample collection and treatment, RNA extraction procedure and resulting RNA quality. Biological variation may arise from sample-to-sample inconsistencies at the cellular level or large changes in the global microRNA population. Both types of variation require normalization of real-time PCR data to one or more endogenous control genes in order to allow correct quantitation of microRNA levels.

Selection of a good control

It is critical and far from trivial to choose the optimal control or combination of controls for normalization of quantitative microRNA analysis. Ideally, the controls

should be empirically validated for each study. No single control can be recommended for use across all types of tissue and cells. Even among the most commonly used housekeeping genes e.g. beta-actin and GAPDH significant variations in expression level between samples can be observed^{ref3}.

Listed below are features that characterize a good endogenous control candidate for normalization of microRNA quantitation:

- Expression at similar level to the microRNAs in the study
- Invariant expression across all samples of the study
- Similar small size as the microRNAs (e.g. similar stability, extraction and quantitation efficiency)
- Not regulated under the experimental conditions

Generally, it is thought that validation of several controls is necessary in order to find the most appropriate candidates for each microRNA quantitation study. Here we evaluate a panel of miRCURY LNA™ Endogenous control primer sets for human and mouse targets, which all meet the above criteria. We further present a commonly used method for normalization of quantitative PCR of microRNAs.

Reliable miRCURY LNA™ Endogenous control primer set

A panel of primer sets (Table 1) have been developed for accurate and sensitive quantitation of small nucleolar RNAs (snoRNAs), U6 snRNA and 5S rRNA when used as endogenous controls in the miRCURY LNA™ microRNA PCR System to normalize microRNA quantitation.

The Endogenous control primer sets have been designed with a gene specific reverse transcription primer in order to reduce non-specific signals to a minimum and to mirror the miRCURY LNA™ microRNA primer sets. The primers have been chosen to work optimally with the miRCURY LNA™ First strand cDNA kit and miRCURY LNA™ SYBR® Green master mix. Figure 1 shows the amplification curves for the 5S rRNA Endogenous control on a dilution series of human breast total RNA. Figure 2 shows cycle numbers for the panel of endogenous controls on a dilution series of the same total RNA. The cycle numbers from the endogenous controls show a linear correlation over a broad range of input RNA.

Table 1

miRCURY LNA™ Endogenous control primer set	Alternative names	Target organism
SNORD38B	U38B; RNU38B	hsa
SNORD44	U44; RNU44	hsa
SNORD48	U48; RNU48	hsa
SNORD49A	U49; U49A; RNU49	hsa
SNORA66	U66; RNU66	hsa
5S rRNA		hsa
U6 snRNA	U6	hsa; mmu

Table 1. miRCURY LNA™ microRNA system, Endogenous control primer sets. For further details, please see www.exiqon.com/pcr

Figure 1

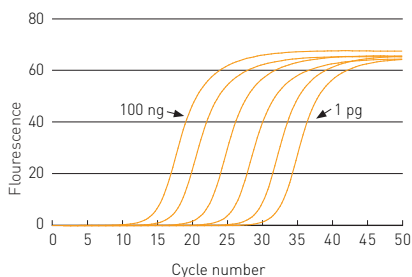


Figure 1. Amplification curves for 5S rRNA from a dilution series of human breast RNA. By using the miRCURY LNA™ PCR System, real-time PCR amplification of the abundant 5S rRNA results in linear read out for serial dilutions (1 pg to 100 ng) of total RNA input with a correlation coefficient $R^2 = 0.997$.

Figure 2

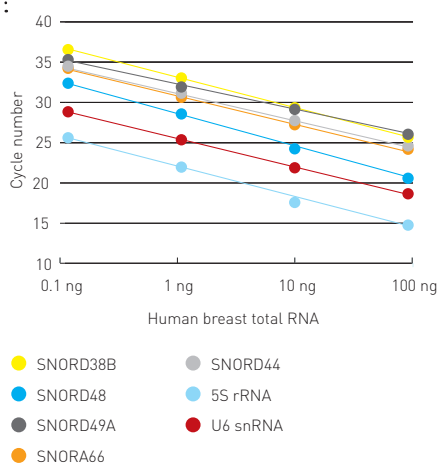


Figure 2. Assay sensitivity. The miRCURY LNA™ PCR System was used for real-time PCR amplification of serial dilutions of total human breast RNA (0.1 ng to 100 ng). All miRCURY LNA™ Endogenous control assays exhibit linear read out with correlation coefficients $R^2 \geq 0.99$.

Constant expression levels of endogenous and microRNA controls across a variety of normal and tumor tissues

Recent studies suggest that some microRNAs may be useful as normalizers in real-time PCR experiments due to their constant expression levels across many different types of tissues and samples^{ref 1}. In addition to the newly developed miRCURY LNA™ Endogenous control assays, we therefore tested primer sets for microRNAs hsa-miR-191 and hsa-miR-103 for their stability in expression level across a panel of normal and tumor tissues. Figure 3 shows the average cycle numbers of each of the assays in ten normal and ten tumor samples as well as two NCI-60 prostate cell lines. Both the endogenous controls and the two chosen microRNAs show relatively stable expression levels across the samples tested indicating their applicability for normalization of microRNA expression levels. However, it is important to note that the expression level of any normalizer should be validated as stable in the samples of each real-time PCR study.

Procedure for normalization of microRNA quantitation

In general it is recommended to employ as many controls as possible to guarantee proper real-time PCR quantitation. Various different programs exist for evaluating the best performing endogenous controls (e.g. GeNorm, NormFinder and BestKeeper) and for applying multiple endogenous controls for normalizing target gene expression (e.g. qBase and REST). Since the method(s) of choice for normalizing real-time PCR data are highly individual, it is recommended to visit the following web-site for detailed information about the approaches, methods and software available for real-time PCR quantitation: www.gene-quantification.info

To illustrate both the importance of selecting the right control for normalization and the advantages of using multiple controls, we have used three of the endogenous controls for quantitation of three microRNA targets in tumor versus normal tissue. In Figure 4A, the controls 5S rRNA, SNORD38B and SNORD44 have been used individually to normalize the expression of three microRNAs. When compared with the results from the same analysis on miRCURY LNA™ Arrays (Figure 4C), it is clear that using SNORD44 for normalization would lead to the incorrect conclusion that hsa-miR-203 is not regulated between the two samples. However, when applying all three controls for normalization using qBasePlus software, both real-time PCR and array show the same trend, confirming the differential expression of all three microRNAs.

One simplified approach for normalizing target gene expression with the miRCURY LNA™ Endogenous controls is to apply the comparative quantitation “ $\Delta\Delta C_T$ ” method also known as the $2^{-\Delta\Delta C_T}$ method^{ref.4}. This relies on comparing the differences in cycle number threshold (C_T -values) obtained for the target(s) and selected control in a sample of interest with the C_T -values obtained in a control sample (e.g. tumor versus normal tissue). Hence,

in order to normalize microRNA expression levels, real-time PCR is performed by using miRCURY LNA™ microRNA primer set(s) of interest and miRCURY LNA™ Endogenous control primer set(s) in parallel. When using this approach, it is critical that the level of the selected control is not regulated by the experimental conditions.

Data is normalized as follows: First the C_T -values for all samples are extracted and the ΔC_T is calculated as the difference in C_T -value between microRNA target and endogenous control:

$$\Delta C_T = C_T (\text{target microRNA}) - C_T (\text{endogenous control})$$

Secondly, the $\Delta\Delta C_T$ is calculated:

$$\Delta\Delta C_T = \Delta C_T (\text{sample of interest}) - \Delta C_T (\text{control sample})$$

Normalization of target gene expression in the sample of interest is determined as:

$$2^{-\Delta\Delta C_T}$$

Finally, the normalized level of expression in the control sample is set to 1 and the change of target expression is determined as: Fold change in target microRNA expression = 1 – normalized target microRNA expression in sample of interest.

Figure 3

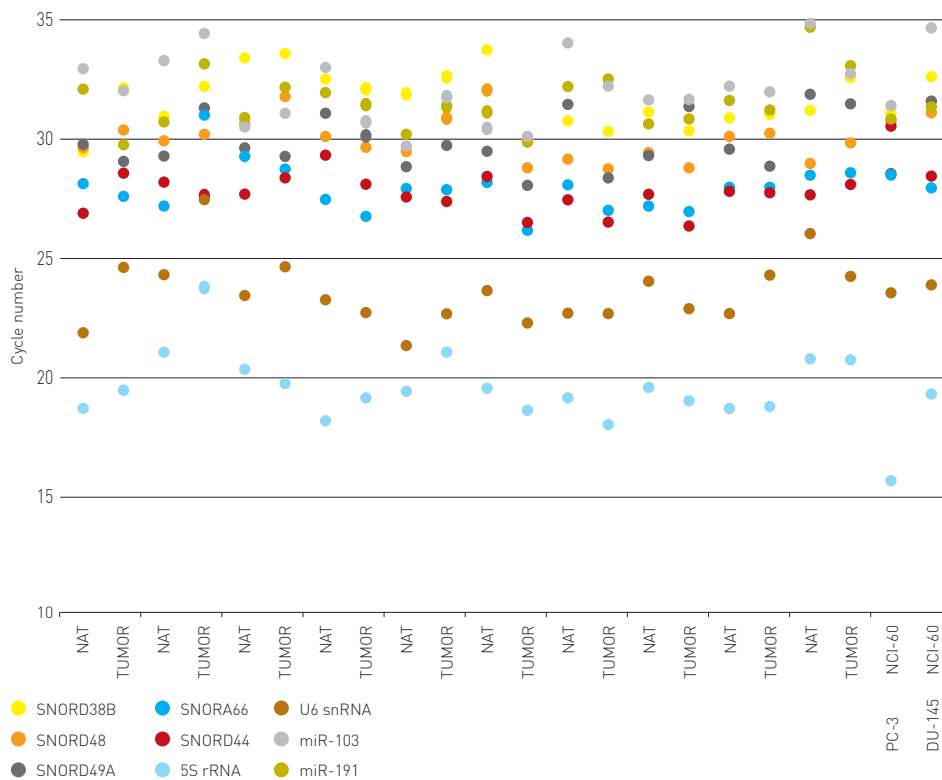


Figure 3. Expression profile of miRCURY LNA™ Endogenous controls across a panel of cancer and normal tissues. Ten ng of total RNA from 10 matched tumor versus normal adjacent tissue (NAT) samples and 2 NCI-60 prostate cell lines were employed for real-time PCR amplification using the miRCURY LNA™ PCR System. Included were primer sets for all miRCURY LNA™ Endogenous controls as well as primer sets for 2 miRCURY LNA™ microRNAs, hsa-miR-103 and hsa-miR-191. Cycle numbers illustrate that expression levels of each of the controls and 2 microRNAs are very similar in various tissues and cell types. N= normal adjacent tissue (NAT); T=tumor tissue; Matched tissue samples include bladder, cervix, esophagus, liver, lung, breast, ovary, prostate, stomach and uterus.

Figure 4

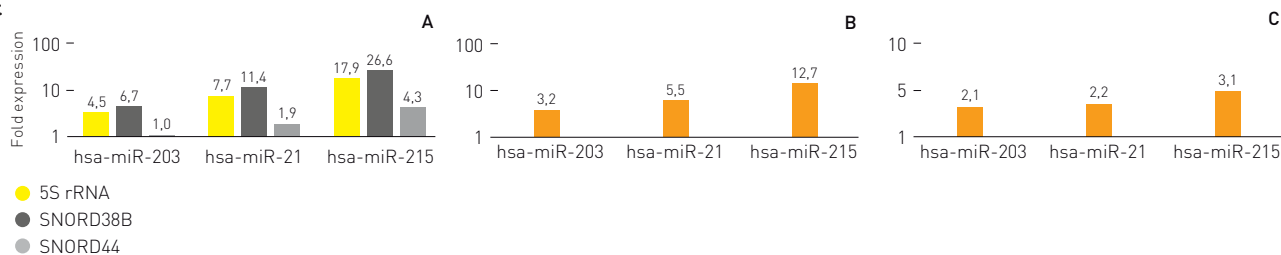


Figure 4. Real-time PCR normalization with miRCURY LNA™ Endogenous controls. Three miRCURY LNA™ Endogenous controls 5S rRNA, SNORD38B and SNORD44 were applied for quantitation of hsa-miR-21, hsa-miR-203 and hsa-miR-215 in an example of matched tumor versus normal adjacent tissues (NAT) **A)** Expression 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. The qBasePlus software was applied for data analysis. **C)** Normalized expression profile of hsa-miR-21, hsa-miR-203 and hsa-miR-215 from miRCURY LNA™ Array analysis. 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.

References

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Ordering information

IMPORTANT: The Universal PCR primer for the real-time PCR step of the protocol is supplied with the miRCURY LNA™ SYBR® Green Master Mix.

Product number	Product name	Product description
201000	miRCURY LNA™ SYBR® Green Master Mix	miRCURY LNA™ microRNA PCR System, SYBR® green master mix (200 rxns). Includes Universal PCR primer and 5S rRNA positive control.
201100	miRCURY LNA™ First-strand cDNA Kit	miRCURY LNA™ microRNA PCR System, First strand cDNA synthesis kit (200 rxns)
202xxx	miRCURY LNA™ microRNA Primer Set	miRCURY LNA™ microRNA PCR System, microRNA primer set (50 RT rxns, 100 PCR rxns)
2015xx	miRCURY LNA™ Endogenous Control Primer Set	miRCURY LNA™ microRNA PCR System, Endogenous Control primer set (50 RT rxns, 100 PCR rxns)