MicroRNA Expression Profiling
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miRNA expression profiling - from reference genes to global mean normalization

Barbara D'haene[1], Pieter Mestdagh[2], Jan Hellemans[1], Jo Vandesompele[1,2]

1 Biogazelle, Zwijnaarde, Belgium
2 Center for Medical Genetics, Ghent University, Ghent, Belgium

Abstract

MicroRNAs (miRNAs) are an important class of gene regulators, acting on several aspects of cellular function such as differentiation, cell cycle control and stemness. These master regulators constitute an invaluable source of biomarkers, and several miRNA signatures correlating with patient diagnosis, prognosis and response to treatment have been identified. Within this exciting field of research, whole-genome RT-qPCR based miRNA profiling in combination with a global mean normalization strategy has proven to be the most sensitive and accurate approach for high-throughput miRNA profiling (Mestdagh et al., Genome Biology, 2009).

In this chapter, we summarize the power of the previously described global mean normalization method in comparison to the multiple reference gene normalization method using the most stably expressed small RNA controls. In addition, we compare the original global mean method to a modified global mean normalization strategy based on the attribution of equal weight to each individual miRNA during normalization. This modified algorithm is implemented in Biogazelle's qbasePLUS software and is presented here for the first time.

Introduction

Accurate quantification of micoRNA (miRNA) gene expression is a major challenge in the field and largely depends on two factors, i.e. the technology used to measure miRNA expression and the choice of a proper normalization strategy. Several methods have been developed to quantify miRNA expression such as microarrays[1-4] bead-based flow-cytometry[5] and small-RNA sequencing[6-9]. While these methods enable genome-wide miRNA expression profiling, they typically require substantial amounts of input RNA which precludes the use of single cells, small biopsies or body fluids such as serum, plasma, urine or sputum. While reverse transcription quantitative PCR (RT-qPCR) intrinsically has a much higher specificity and sensitivity, down to a single molecule, it requires some adjustments to enable quantification of small RNA molecules such as miRNAs. Mature miRNAs consist of 21-25 nucleotides and are too short to serve as templates in a RT-qPCR reaction. Therefore, different modifications of the classical RT-qPCR workflow have been developed in order to allow RT-qPCR-based miRNA expression profiling. One approach relies on the use of a miRNA specific stem-loop primer that hybridizes to the 3' end of the mature miRNA[10]. The loop unfolds upon denaturation, providing an elongated template that can be used in a subsequent qPCR-reaction. Alternatively, the mature miRNA is polyadenylated and a poly-T primer is used to initiate the RT-reaction[11]. Next to sensitivity, RT-qPCR based approaches have a superior specificity, linear dynamic range of quantification and a high level of flexibility making RT-qPCR the gold standard for small RNA expression profiling. Importantly, the accuracy of the results obtained through RT-qPCR miRNA expression profiling is largely dependent on a proper normalization strategy[12, 13]. Different variables, inherent to the RT-qPCR workflow need to be controlled for in order to distinguish true biological changes from technical variation. These include the amount of starting material, enzymatic efficiencies, and overall transcriptional activity[14]. The use of multiple stable reference genes is generally accepted as the method of choice for RT-qPCR data normalization[14]. These stable reference genes can be identified from a set of candidate reference genes in a pilot experiment on a selection of samples that are representative for the experimental conditions under investigation. Different algorithms, such as geNorm, allow to rank the candidate reference genes according to their stability and indicate the optimal number of reference genes required for accurate normalization of gene expression. In the case of miRNA expression profiling, only few candidate reference miRNAs have been identified[15]. Typically, other small endogenous non-coding RNAs such as small nuclear (U6) and small nucleolar (U24, U26) RNAs have been used.

In 2009, Mestdagh et al.[13] introduced the global mean normalization method to normalize data from RT-qPCR miRNA profiling studies in which a large number of miRNAs are tested per sample (e.g. whole miRNome). This method outperformed other normalization strategies commonly used at the time (e.g. multiple target reference normalization using endogenous small RNA controls) and it has been considered the gold standard method since.

Normalizing genome-wide miRNA expression data – the global mean

The global mean normalization method consists of three successive steps. First, all Cq values above a certain threshold are considered noise and are discarded from further analysis (based on an 14-cycle sample pre-amplification procedure following mageplex reverse transcription, we routinely use 32 as cut-off value). The arithmetic average Cq value is then calculated for each individual sample and subsequently subtracted from each individual Cq value for that sample. The procedure results in normalized expression values in logE scale (E being the base of the exponential amplification function, with 2 being a good estimate); the more negative, the higher a particular miRNA is expressed (Table 1).

Table 1
Normalized expression values of a particular miRNA in 2 samples (log scale, global mean normalization)

	Sample 1	Sample 2
hsa-let-7a	-1.50	-2.32

- Sample 1: the fold difference of let-7a compared to the mean Cq = $2^{-(-1.50)} = 2.83^{*}$
  - Sample1: Let-7a is 2.83 times higher expressed than the mean.
- Fold change for let-7a of sample 1 relative to sample 2 = $2^{-[-1.507-(-2.32)]} = 0.57$
- Fold change for let-7a of sample 2 relative to sample 1= $2^{-[-2.32-(-1.50)]} = 1.76$

[*] Assuming equal Cq values for equal transcript numbers

In this chapter, we summarize the power of the previously described global mean normalization method in comparison to the multiple reference gene normalization method using the most stably expressed small RNA controls (Table 2). In addition, we compare the original global mean method to a modified global mean normalization strategy based on the attribution of equal weight to each individual miRNA during normalization. This modified algorithm is implemented in Biogazelle's qbase^{PLUS} software as of version 2.0 (http://www.qbaseplus.com) and is presented here for the first time.

The modified global mean normalization strategy conveniently generates normalized relative quantities (NRQ values) in a linear scale. These values can be obtained using four simple steps in qbase^{PLUS}: (1) auto-exclusion of miRNAs below a certain expression level, (2) conversion of Cq values into relative quantities (RQs), (3) calculation of sample specific normalization factor (NF) as the geometric mean of the RQs of all expressed targets per sample, and (4) conversion of RQs into normalized RQs (NRQs) by dividing the RQs by the sample specific NF. Steps 2-4 are simultaneously performed if the user selects one of the two available global mean normalization strategies. The NF can be calculated based on the RQs of all expressed targets in the sample for which the normalization is determined without taking into account the other samples ('modified global mean normalization'). Alternatively, the normalization factor is calculated based on the RQs of the targets that are expressed in all samples ('modified global mean normalization on common targets').

The different strategies to normalize large scale RT-qPCR miRNA profiling data are compared in a comprehensive manner by reanalysis of published datasets[13, 16]. These datasets include expression profiles for 430 miRNAs and 18 controls in 61 neuroblastoma (NB) tumor samples, 366 miRNAs and 18 controls in 49 T-cell acute lymphoblastic leukemia (T-ALL) samples and 636 miRNAs and 19 controls in 32 cell-free sputum samples from never smokers, smokers with COPD and smokers without airflow limitation.
The performance of the different normalization strategies is assessed by: (1) evaluating their ability to reduce the overall variation, (2) determining their power to extract true biological variation, and (3) estimating the ability to reduce the number of false positive and false negative calls.

Table 2. Stably expressed small RNA controls used as reference genes

Neuroblastoma	T-ALL	Sputum (non-)smokers
RNU24	RNU24	U6*
RNU44	RNU44	
RNU58A	RNU48	
RNU6B	RNU58A	
	U18	
	Z30	

* Only U6 was found to be expressed in all sputum samples

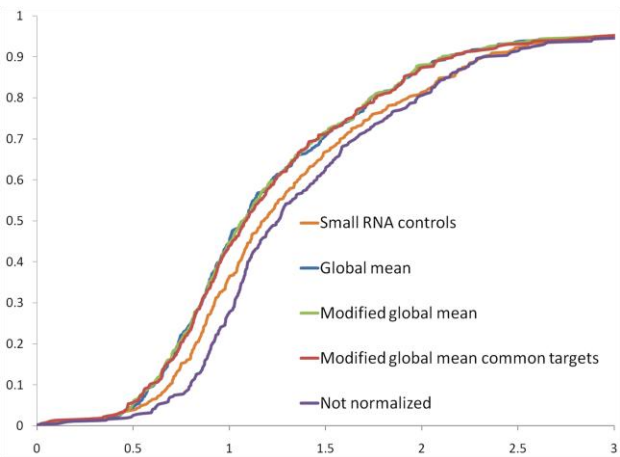
(1) Evaluation of the ability to reduce the overall experimental variation

Any variation in gene expression levels is composed of both true biological and experimentally induced (technical) variation. The purpose of normalization is to reduce the technical variation within a dataset, enabling a better appreciation of the biological variation.

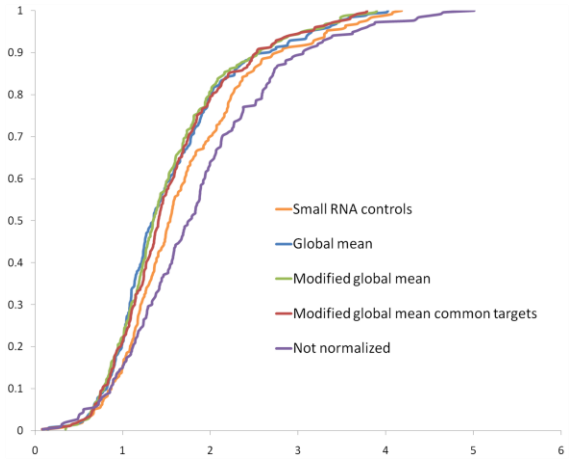
To measure normalization performance, we calculated the standard deviation (SD) for each individual miRNA across all samples within a given dataset upon applying different normalization procedures. Lower standard deviations denote better removal of experimentally induced noise. Cumulative distributions of the SDs for not normalized and normalized data using different methods allows comparison of different normalization methods (Figure 1).

Figure 1. Cumulative distribution of the SD values

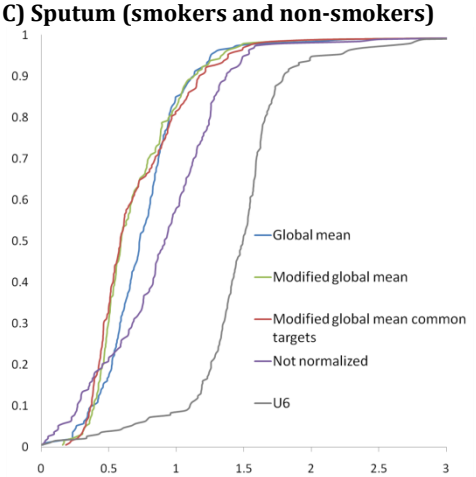
A) Neuroblastoma



B) T-cell acute lymphoblastic leukemia (T-ALL)



C) Sputum (smokers and non-smokers)



Standard deviations (SDs) for each individual miRNA were calculated on log2 transformed relative expression levels normalized with either (1) the original global mean normalization approach, (2) the modified global mean normalization, (3) the modified global mean normalization based on common targets, (4) the multiple reference gene normalization using stably expressed small RNA controls. Standard deviations are also presented for not normalized expression data.

A shift to the left is discernable for all four normalization methods compared to not-normalized data, pointing at the intended reduction of the overall variation. The decrease in variation is least pronounced for multiple reference gene normalization using stable small RNA controls. The latter approach results in a small decrease of the SD values in the NB sample set for the 80% least variable miRNAs. In the T-ALL sample set, it results in a pronounced decrease of the SD values for the 80% most variable miRNAs. These observations indicate that elimination of technical variation is not effective for all miRNAs. Normalization using a single small RNA control (U6) in the sputum sample set even results in an increase of overall variability. In contrast, all three global mean normalization methods result in an overall decrease in variation that is (1) more pronounced compared to stable small RNA control normalization and (2) effective for all miRNAs that are measured.

In conclusion, all three global mean normalization based methods are equally well suited to reduce the technical variation and outperform multiple reference gene normalization using stable small RNA controls.

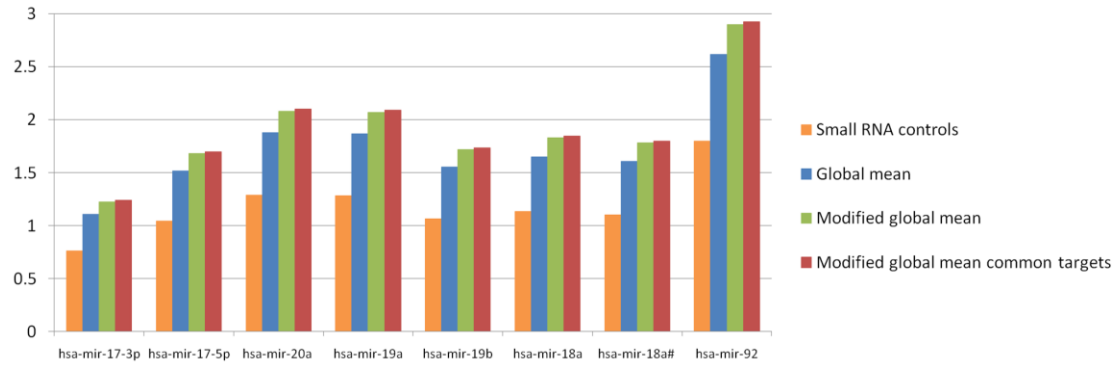
(2) Determination of the power to extract true biological variation

Good normalization approaches should not only reduce the technical variation as much as possible, they should also accentuate true-biological differences.

To assess the impact on appreciation of true biological differences, we evaluated the differential expression of the miRNAs belonging to the oncogenic mir-17-92 cluster in the NB data set. This cluster contains six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92) that are known to be upregulated in NB tumors with MYCN amplification (MNA) in comparison to samples with a normal MYCN copy number (MYCN single copy, MNSC).

The average fold change of the mir-17-92 cluster elements in MNA (n=22) compared to MNSC (n=39) samples was calculated upon normalization using either (1) the original global mean normalization approach, (2) the modified global mean normalization, (3) the modified global mean normalization based on common targets, (4) the multiple reference gene normalization using stable small RNA controls (Figure 2). Normalized results were first log transformed, followed by calculation of the difference between the mean MNA and MNSC group values and exponentiation (anti-log) of the difference.

Figure 2. miR-17-92 upregulation in MYCN amplified neuroblastoma



Average fold change expression (linear scale) difference of six miRNAs residing within the miR-17-92 cluster in MYCN amplified neuroblastoma samples compared to MYCN single copy neuroblastoma samples. Fold changes were calculated upon data normalization with (1) the original global mean normalization approach, (2) the modified global mean normalization, (3) the modified global mean normalization based on common targets, (4) the multiple reference gene normalization using small RNA controls.

When the data are normalized using the small RNA controls, only one out of eight miRNA genes within the miR-17-92 cluster reaches a 1.5-fold expression difference. In contrast, when the data are normalized using one of the global mean normalization methods seven out of eight miRNA transcripts reach at least a 1.5-fold expression difference. Both modified global mean normalization approaches result in true expression differences that are more pronounced in comparison to the original global mean normalization method; three out of eight miRNA transcripts reach a 2-fold expression difference as opposed to one out of eight, respectively.

In conclusion, both the modified global mean normalization and the modified global mean normalization based on common targets perform slightly better than the original global mean normalization and clearly outperform the multiple reference gene normalization strategy using small RNA controls in appreciating true biological differences.

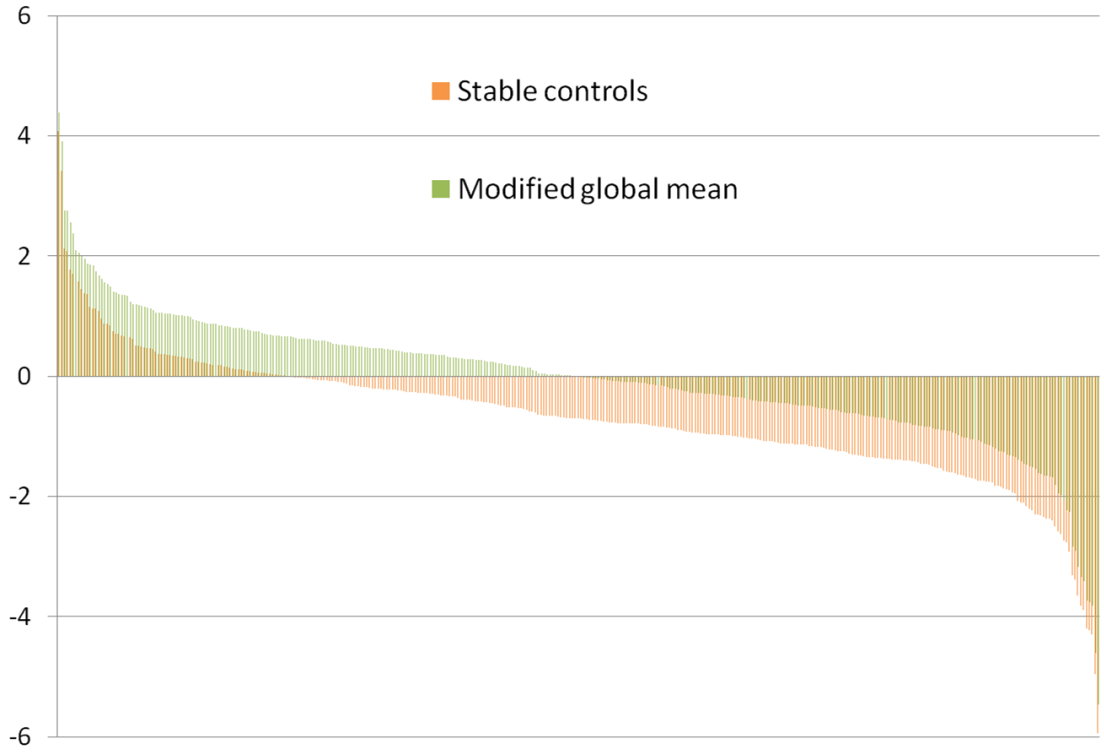
(3) Balancing up and down regulated genes

A fair assumption in most transcriptome-wide gene expression studies is that the number of up- and downregulated genes is approximately equal. To date, there is no biological evidence that the number of downregulated miRNAs is different from the number of upregulated miRNAs in MYCN

amplified neuroblastoma samples compared to MYCN single copy neuroblastoma samples and vice versa. It is hence acceptable to assume the existence of a balanced situation with an equal number of up- and downregulated miRNAs.

The overall differential miRNA expression in the two NB tumor sample subsets may give us an estimate of the ability of the normalization methods to reduce the number of false positives and negatives (Figure 3, Table 3).

Figure 3. Average fold change expression difference of each miRNA in neuroblastoma with respect to the MYCN amplification status



Average fold change expression difference (log2 scale) of each miRNA with a Cq value below 32 cycles in MYCN amplified neuroblastoma samples compared to MYCN single copy neuroblastoma samples (differences calculated as explained for the miR-17-92 cluster in Figure 2).
Fold changes were calculated upon small RNA control normalization (orange) or modified global mean normalization (green). Fold changes are plotted in a log2 scale and sorted from positive (upregulated in MYCN amplified tumor samples) to negative (downregulated in MYCN amplified tumor samples).

Table 3. Number of up and down regulated miRNAs in neuroblastoma with respect to the MYCN amplification status

Normalization	# up regulated	# down regulated
Small RNA controls	80	285
Original global mean	165	200
Modified global mean	180	185
Modified global mean common targets	181	184

Table 4. Number of up and down regulated miRNAs in sputum sample set (smokers and non-smokers) with respect to the smoker status

Normalization	# up regulated	# down regulated
Small RNA controls (U6)	75	85
Original global mean	41	119
Modified global mean	70	90
Modified global mean common targets	72	88

Normalization with small RNA controls suggests that most miRNAs are downregulated in MYCN amplified neuroblastoma samples. The global mean normalization based methods result in a more balanced situation with an approximately equal number of up and down regulated miRNAs.

Normalization with the original global mean normalization methods suggests that most miRNAs are downregulated in sputum samples from smokers (Table 4). The modified global mean normalization based methods result in a more balanced situation with an approximately equal number of up and down regulated miRNAs.

In conclusion, the modified global mean normalization based methods result in a more balanced situation in which the number of down regulated miRNAs equals the number of up regulated miRNAs in neuroblastoma with respect to the MYCN amplification status. This clearly suggests that they have more power to reduce the number of false positives and false negatives in comparison to the multiple reference gene normalization using small RNA controls.

Considering all the results, it is fair to state that the modified global mean normalization methods are slightly better in terms of better appreciation of true biological changes. As biologically relevant miRNA expression differences can be quite small, we highly recommend applying the modified normalization method to obtain more accurate results for RT-qPCR miRNA profiling studies. The impact of non-random missing data on calculating a global mean is reduced by removing the effect of differential expression between genes when calculating the modified global mean on relative quantities.

Multiple reference gene normalization using small RNAs that resemble the global mean

The global mean normalization methods are only valid for miRNA profiling studies in which a large number and unbiased set of genes are measured. This is because these methods are based on two assumptions: (1) only a minority of miRNAs is differentially expressed, and (2) the number of down regulated miRNAs is balanced against the number of up regulated miRNAs.

Whole miRNome studies (using RT-qPCR, microarrays, or next-generation sequencing) often serve as a starting point in a pilot screen to identify differentially expressed miRNAs in certain subsets of samples. Subsequent studies on much larger sample groups are required to validate the statistical significance and to assess the biological relevance of the regulated miRNAs. In such RT-qPCR validation studies in which the expression of only a handful of miRNAs is measured, it is not valid to use a global mean normalization method.

To overcome this problem, Mestdagh et al.[13] proposed to use multiple stably expressed miRNAs or small RNA controls, identified by an expression pattern similar to the global mean level, referred to as genes resembling the mean expression value.
Such reference genes can be identified from prior whole miRNome expression profiling studies or from a pilot experiment specifically performed to identify stably expressed genes resembling the mean. The results from Mestdagh et al.[13] indicate that a normalization factor based on the

selection of miRNAs/small RNA controls resembling the mean expression value performs equally well compared to the mean expression value itself.

Identification of such stably expressed reference miRNA/small RNA genes consists of two steps. First, candidate miRNAs/small RNA controls that resemble the mean expression value are identified, followed by selection of the most stably expressed reference from this group in a second step. In brief, the standard deviation (SD) for each individual miRNA across all samples is calculated after global mean normalization. The optimal set and number of miRNAs/small RNA controls for normalization is then determined through genorm^{PLUS} analysis of the ten best ranked candidate reference genes, being miRNAs or small RNA controls with the smallest standard deviation. To avoid the possibility of including co-regulated miRNAs in the genorm^{PLUS} analysis, miRNAs residing within the same gene cluster should be excluded, hereby retaining only one miRNA per cluster.

For publically available RT-qPCR data, the miRNA body map enables selection of stably expressed miRNAs/small RNAs reference genes using the strategy outlined above (http://www.mirnabodymap.org).

Multiple reference gene normalization using stably expressed small RNAs

If no prior whole miRNome expression profiling can be performed to identify genes whose expression pattern resembles the mean expression value, then a careful selection of the most stable small RNA controls should be performed in a typical geNorm pilot experiment[14].
Such a pilot experiment involves the analysis of a set of candidate reference small RNAs (preferentially more than 8, each belonging to a different family (e.g. RNU X is not a good choice if RNU Y is already selected as candidate reference RNA) on a representative sample set (at least 10 independent samples). A geNorm analysis determines the expression stability value for each gene (M value) and it calculates normalization factor V values. Both values are subsequently used to determine the optimal number and set of reference RNAs to be used in further studies.
A useful tool is the genorm^{PLUS} module incorporated into qbase^{PLUS}, a substantial improvement over the old geNorm version running in Microsoft Excel, in terms of handling missing data, subranking of the best reference gene pair, fully automating the calculations and providing interpretation of the results. The genorm^{PLUS} expert report enables straightforward interpretation as it provides recommendations on the number and nature of genes to be used for optimal normalization. In addition, it compares the stability of the proposed reference genes against empirically determined reference gene values[17].
Of note, Peltier and Latham[15] reported 2 miRNAs, miR-103 and miR-191, that were stably expressed in different normal and cancer tissues, suggesting that they may serve as universal reference miRNAs. In contrast, the reference miRNAs identified by Mestdagh et al.[13] varied substantially between different datasets. While miRNAs that are stably expressed across different tissues may exist, it is highly recommended to evaluate their stability in a selection of samples, representative for the entire sample set in the study.

Please note that results obtained through normalization with small RNAs should be interpreted with care. The side-by-side comparison of small RNA normalization with global mean normalization indicated that small RNAs are less efficient in reducing the technical variation and do not result in balanced expression differences. As biologically relevant miRNA expression differences can be quite small, it is not unconceivable that interesting expression differences will be missed.

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References

1. Castoldi, M. et al. A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). *RNA* **12**, 913-920 (2006).
2. Liu, C.G. et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci U S A* **101**, 9740-9744 (2004).
3. Nelson, P.T. et al. Microarray-based, high-throughput gene expression profiling of microRNAs. *Nat Methods* **1**, 155-161 (2004).
4. Sioud, M. & Rosok, O. Profiling microRNA expression using sensitive cDNA probes and filter arrays. *Biotechniques* **37**, 574-576, 578-580 (2004).
5. Lu, J. et al. MicroRNA expression profiles classify human cancers. *Nature* **435**, 834-838 (2005).
6. Kuchenbauer, F. et al. In-depth characterization of the microRNA transcriptome in a leukemia progression model. *Genome Res* **18**, 1787-1797 (2008).
7. Morin, R.D. et al. Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. *Genome Res* **18**, 610-621 (2008).
8. Ramsingh, G. et al. Complete characterization of the microRNAome in a patient with acute myeloid leukemia. *Blood* **116**, 5316-5326 (2010).
9. Schulte, J.H. et al. Deep sequencing reveals differential expression of microRNAs in favorable versus unfavorable neuroblastoma. *Nucleic Acids Res* **38**, 5919-5928 (2010).
10. Chen, C. et al. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* **33**, e179 (2005).
11. Shi, R. & Chiang, V.L. Facile means for quantifying microRNA expression by real-time PCR. *Biotechniques* **39**, 519-525 (2005).
12. Brattelid, T. et al. The Normalization Strategy is Critical for the Outcome of miRNA Expression Analyses in the Rat Heart. *Physiol Genomics* (2010).
13. Mestdagh, P. et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* **10**, R64 (2009).
14. Vandesompele, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034 (2002).
15. Peltier, H.J. & Latham, G.J. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA* **14**, 844-852 (2008).
16. Van Pottelberge, G.R. et al. MicroRNA Expression in Induced Sputum of Smokers and Patients with Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* (2010).
17. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. & Vandesompele, J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* **8**, R19 (2007).