Normalization matters: normalizer election effects on sperm miRNA quantification

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INTRODUCTION

- Mean Centering Restricted (**MCR**) is considered the most reliable normalization method for high-throughput **qRT-PCR miRNA profiling in human spermatozoa**. It is based on the normalization by the mean Ct value of miRNAs expressed in all samples[1].

- Appropriate reference controls still need to be established in sperm singleplex quantifications, since the validation of control molecules is highly suggested for every specific cell type or tissue[2].

- Concordance Correlation Restricted (CCR) algorithm is designed to select the miRNAs with the highest approximation to MCR normalizing value, so that they can be extrapolated to assays including the analysis of a low number of miRNAs[1]. Additionally, RNU6B is frequently used as normalizer in the literature.

Objective: to provide the best candidates as normalizers for sperm miRNA qRT-PCR singleplex analyses.

Materials and Methods

qRT-PCR analysis of sperm samples [3,4]

- **Fertile population (n=10)**
- **Infertile population (n=38)**
  - Teratozoospermic
  - Oligozoospermic
  - Astenozoospermic
  - Normozoospermic

Normalizing strategies

Reference method: MCR

Tested methods:

- Small nuclear RNA (selected from literature): RNU6B
- 4 miRNAs (selected by CCR): miR-100-5p, miR-146b-5p, miR-92a-3p, miR-30a-5p
- Combination of the best 2 normalizers: miR-100-5p, miR-30a-5p

Validated parameters

- **Ubiquity**: 15 ≤ Ct ≤ 35 in all samples
- **Expression stability**: Variance analysis
- **Homogeneity among populations**: Nonparametric Wilcoxon test (P<0.01, FDR correction)

MCR comparative analyses

1. Differentially-expressed miRNAs → Receiver Operating Characteristic (ROC) curves
2. Predicted target genes
3. Enriched biological Processes

Based on:
- True Positives (TPs)
- False Positives (FPs)
- False Negatives (FNs)

Results

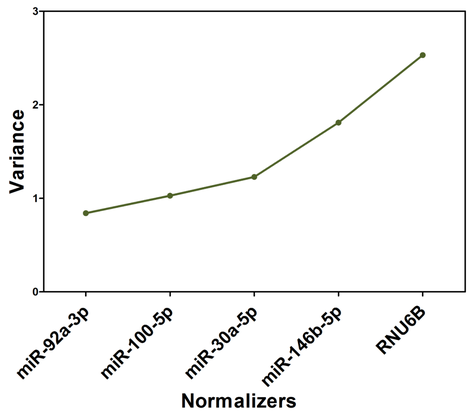
Validated parameters

Ubiquity

miR-100-5p	✓
miR-146b-5p	✓
miR-92a-3p	✓
miR-30a-5p	✓
RNU6B	✓

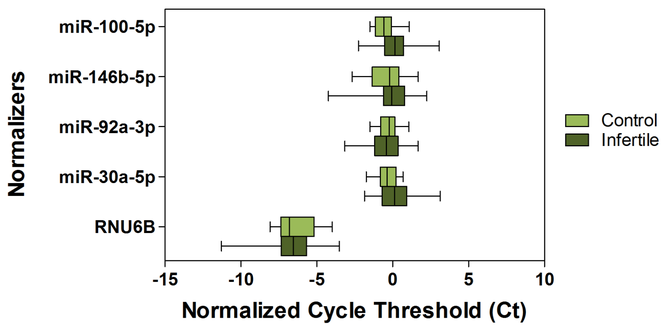
Every tested normalizer was expressed in all analyzed samples

Expression stability



Variance value is inversely proportional to expression stability. RNU6B and miR-146b were the most unstable normalizers whereas miR-92a, miR-100 and miR-30a showed the highest stability values

Homogeneity among fertile and infertile populations

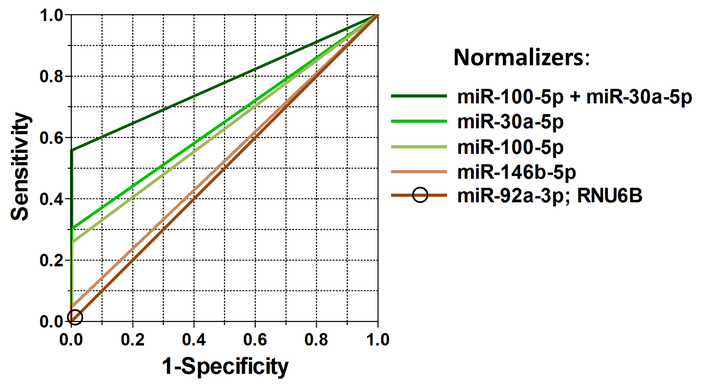


Expression levels of each normalizer in the control and infertile populations was evaluated by a Nonparametric Wilcoxon test. No differential expression was found to be statistically significant.

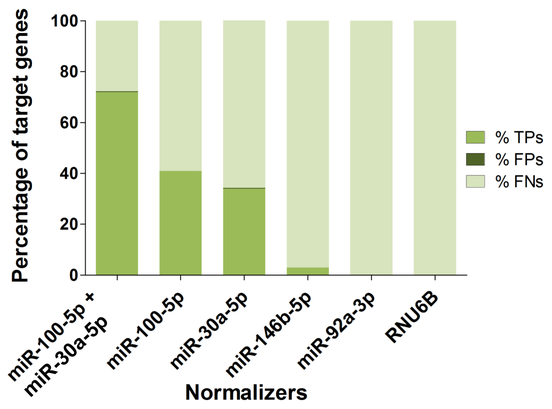
MCR comparative analyses

ROC curves from the analysis of differentially-expressed miRNAs

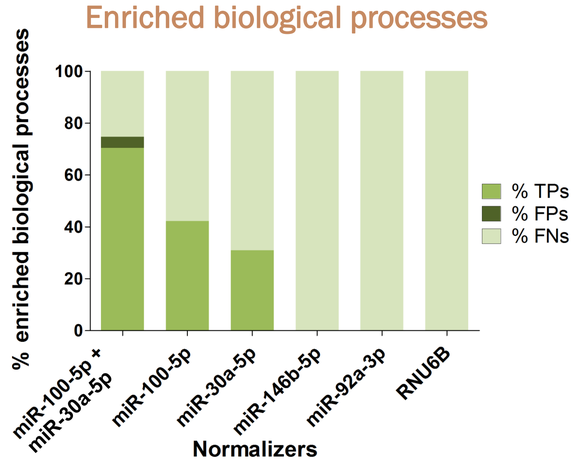
A higher Area Under the Curve indicates a better proximity to MCR regarding differentially-expressed miRNAs.
Circumferences along the Y axis and in the legend remark the presence of two overlapping ROC curves.



Predicted target genes



Enriched biological processes



Percentages of TPs, FPs and FNs of predicted target genes (left) and enriched biological processes (right) for each normalization method in comparison with the results obtained from data normalized by MCR.

CONCLUSIONS

- **RNU6B** is **inappropriate to normalize sperm miRNA expression data; it** shows the lowest expression stability, and a scant proximity to MCR results.
- The combination of **miR-100-5p and miR-30a-5p** is the **best normalization strategy** for sperm miRNA qRT-PCR singleplex analyses. Both molecules are ubiquitous, stably expressed, and homogeneous through different populations, besides data normalized by this method displays the highest rates of proximity to MCR method.

REFERENCES

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