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Nucleic Acids Research Group 2010-2011 Study

Benchmarking miRNA Expression Levels in Degraded RNA Samples Using Real-Time RT-qPCR and Microarray Technologies

J Holbrook[1], S Chittur[2], S Tighe[3], V Nadella[4], R Carmical[5], K Sol-Church[1], AT Yeung[6]
[1]Nemours/A.I. duPont Hospital for Children, [2]University at Albany-SUNY, [3]University of Vermont, [4]Ohio University, [5]University of Texas Medical Branch, [6]Fox Chase Cancer Center.

NUCLEIC ACIDS RESEARCH GROUP

INTRODUCTION

The Nucleic Acid Research Group (NARG) study has evaluated the impact of RNA integrity on priming strategies for the detection of nine miRNA targets, selected based on Microarray Research Group (MARG) data, using Real-Time RT-qPCR. These miRNA targets represent groups that are expressed at low, medium, or high levels in the First Choice human brain reference RNA sample. The two RT-qPCR priming strategies tested in this study include the miRNA TaqMan assay (MegaPlex) from ABI and the RT²miRNA qPCR assay from Qiagen/SABiosciences. The samples used as template have been subjected to controlled degradation using RNase A to RNA Integrity Number (RIN) values of 8 (good), 4 (moderately degraded), and 2 (severely degraded). In addition, the same RNA templates were further analyzed using universal poly (A) tailing and labeling followed by hybridization to Affymetrix miRNA GeneChips. We present our findings of the effects of RNA degradation on RT priming strategies for miRNA detection, contrasts of qPCR results obtained using different technologies.

RESEARCH PLAN

- **To compare target specific stem loop structure and poly-A tagging methods for miRNA quantification by QPCR**
- **To investigate the effects of RNA integrity on miRNA quantification**
- **Compare RT-qPCR results to microarray data**

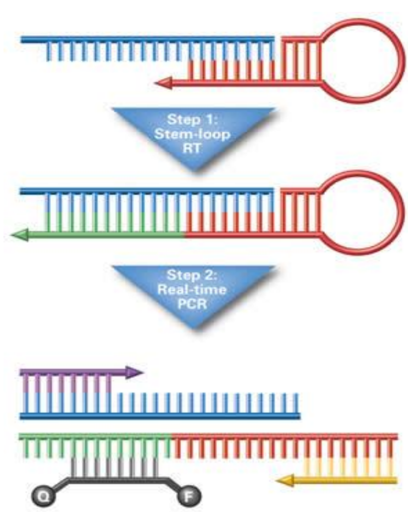
miRNA LIST

miRNA name	copy number *	mature miRNA sequence	ABI Assay ID	Qiagen Catalog Number	mirBASE 14.0 Accession number
hsa-miR-122a	1.3×10^3	UGGAGUGUGACAAUGGUGUUUG	000445	MPH00020A	MIMAT0000421
hsa-miR-192	8.9×10^3	CUGACCUAUGAAUUGACAGCC	000491	MPH00080A	MIMAT0000222
hsa-miR-194	1.3×10^4	UGUAACAGCAACUCCAUGUGGA	000493	MPH00083A	MIMAT0000460
hsa-miR-370	7.6×10^4	GCCUGCUGGGGUGGAACCUGGU	000558	MPH00185A	MIMAT0000722
hsa-miR-19a	1.6×10^5	UGUGCAAAUCUAUGCAAAACUGA	000395	MPH01214A	MIMAT0000073
hsa-miR-382	1.8×10^5	GAAGUUGUUCGUGGUGGAUUCG	000572	MPH00201A	MIMAT0000737
hsa-miR-135a	3.2×10^5	UAUGGCUUUUUAUUCCUAUGUGA	000460	MPH00035A	MIMAT0000428
hsa-miR-149	1.2×10^6	UCUGGCUCCGUGUCUUCACUCCC	000472	MPH00053A	MIMAT0000450
hsa-miR-9a	9.3×10^7	UCUUUGGUUAUCUAGCUGUAUGA	000583	MPH00456A	MIMAT0000441

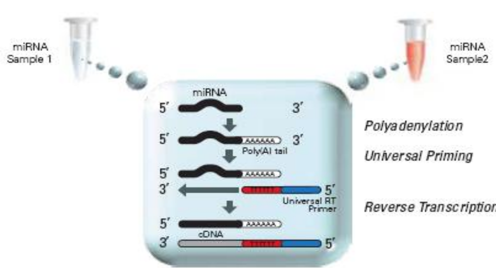
* source: miRNAmap 2.0

METHODS

Target specific stem-loop structure and reverse transcription primer method



PolyA tailing with universal transcription method



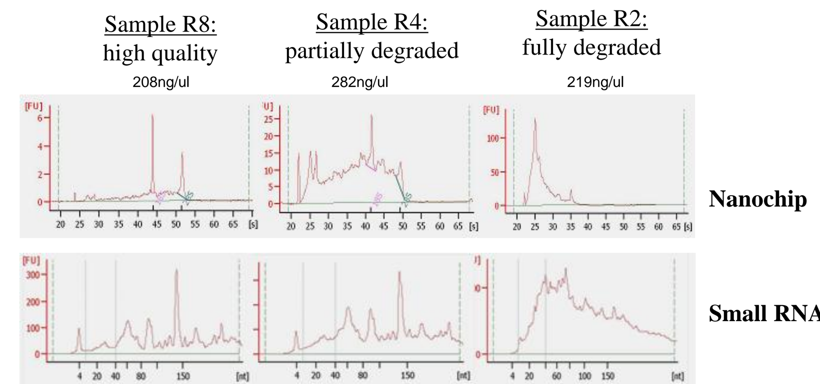
Affymetrix miRNA GeneChips

- **500 ng total RNA input for RIN RNA**
- **Hybridized to the miRNA galaxy arrays using standard labeling procedures with the Genisphere FlashTag HSR kit.**
- **Uses a Poly A polymerase method similar to SAB/Qiagen qPCR**
- **All labs used the same standard input volume that was used for RT-qPCR**
- **Data analyzed using Bioconductor**

METHODS

RNA: Ambion's First Choice Brain Reference RNA (same RNA that was used in previous NARG studies) was degraded using RNase to RIN values of 8 (control), 4, and 2 and stopped with RNase inhibitor. RNA quantity and quality was confirmed using the Nanodrop spectrophotometer and Agilent 2100 Bioanalyzer, respectively.

Sample R8: high quality 208ng/ul | Sample R4: partially degraded 282ng/ul | Sample R2: fully degraded 219ng/ul



Reference RNA Sample RIN- 8, 4, and 2
500 ng Total RNA

↓

Target specific stem-loop structure and reverse-transcription primer

Reverse Transcriptase reaction performed using the ABI TaqMan® MicroRNA Reverse Transcription kit components and the MegaPlex RT primers, no pre-amplification, in duplicate, and with and without RT primer.

AND

PolyA tailing with universal transcription

Reverse Transcriptase reaction performed using the SABiosciences/Qiagen RT² miRNA First Strand Kit, in duplicate, with and without RT primer

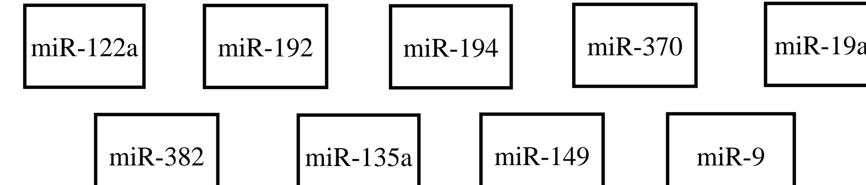
↓

Duplicate PCR reactions on each RT reaction were performed using both the ABI TaqMan Assays and the SAB RT² Assays, specific for the 9 miRNA of interest, with the ABI TaqMan Master Mix or SAB/Qiagen RT² Master Mix-SYBR Green, respectively.

384 well plate, 10ul rxn volume, and cycled using an ABI 7900HT under recommended conditions

OR

96 well plate, 25ul rxn volume and cycled using Stratagene MX3000P or ABI 7500fast instrument specific recommended conditions.



ANALYSIS

Statistical Analysis Methods

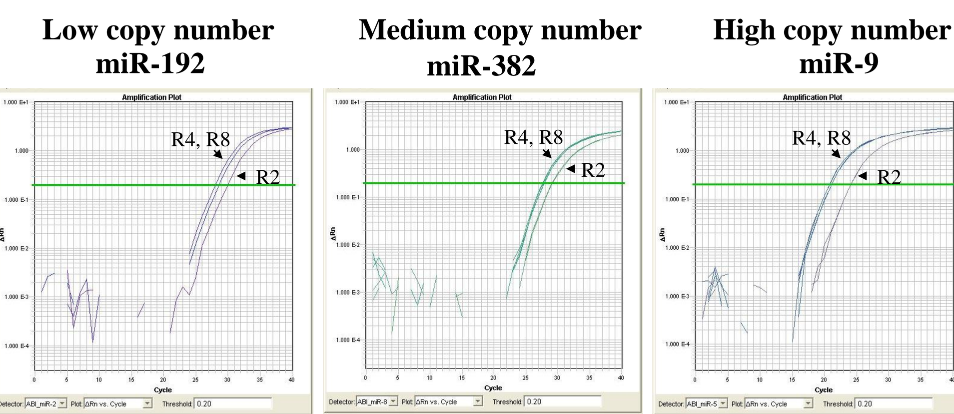
- Average C_q value calculated for each sample (R8, R4, R2).
- ΔC_q values were determined by subtracting the average C_q value of R4 and R2 from the average R8 C_q value (i.e., C_qR4–C_qR8 *OR* C_qR2–C_qR8).
- Principal component analysis performed by Partek Genomics Suite
- Heat maps generated by R_Statistics software
- Microarray data was analyzed without normalization to avoid possible false positive data shift.

STUDY RESULTS

Representative amplification plots for a low, medium and high copy number microRNA

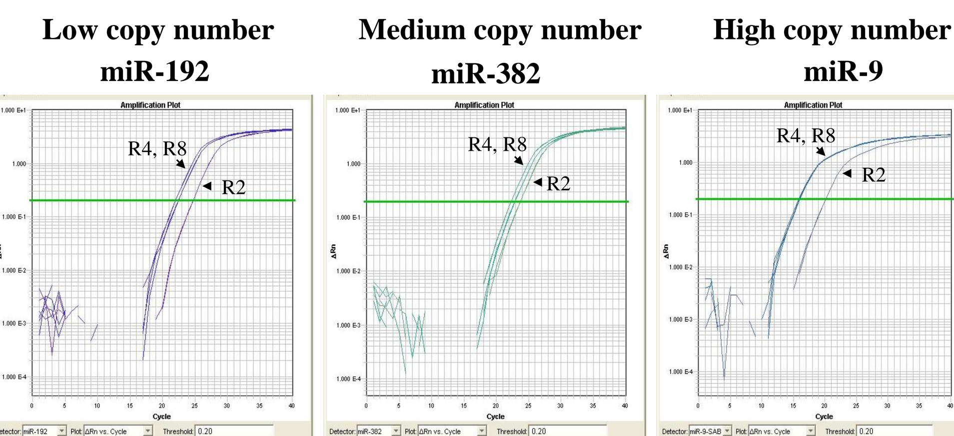
Stem-loop method - TaqMan

Low copy number miR-192	Medium copy number miR-382	High copy number miR-9



PolyA tailing method - SYBR

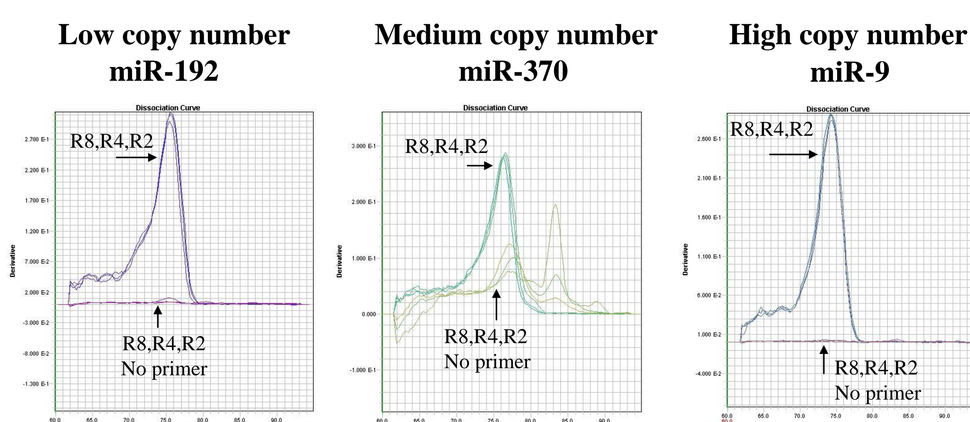
Low copy number miR-192	Medium copy number miR-382	High copy number miR-9



SYBR method dissociation curves

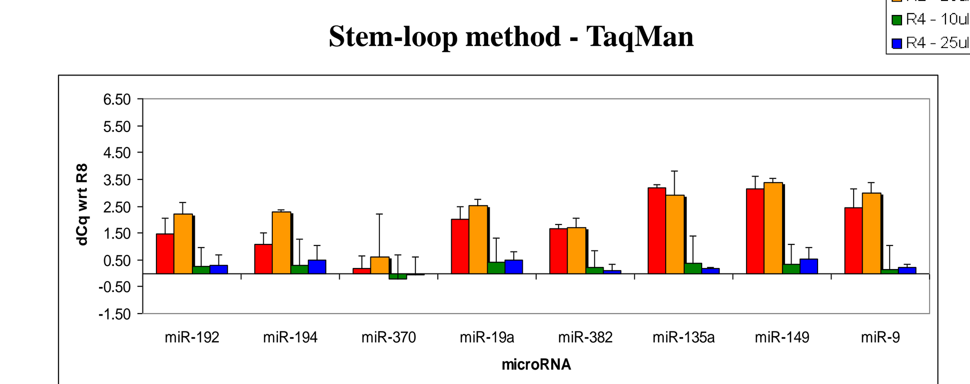
miR-370 was the only miRNA that showed any dissociation curve issues.

Low copy number miR-192	Medium copy number miR-370	High copy number miR-9

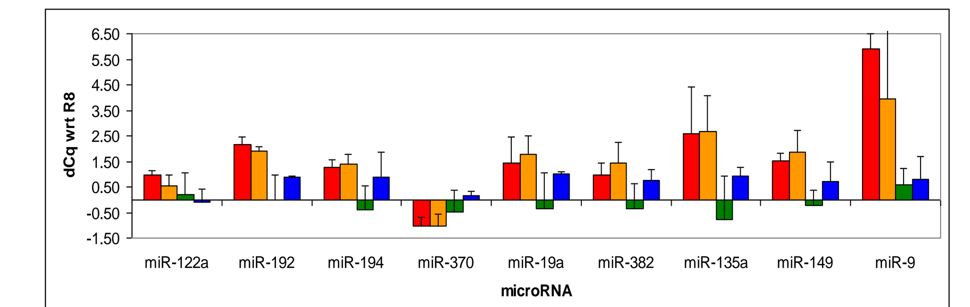


ΔCq vs. miRNA for both methods

Stem-loop method - TaqMan



Poly-A tailing method – SYBR Green



10 ul rxn were performed in a 384 well plate using the ABI 7900HT . 25 ul rxn were performed in a 96 well plate using either the ABI 7500 fast or Stratagene MX3000P.

ΔCq of each miRNA per method for each participating laboratory

Principal Component Analysis

Delineation by method

R2-TaqMan
R2-SYBR
R4-TaqMan
R4-SYBR
R8-TaqMan
R8-SYBR

Delineation by sample and corresponding heat maps

PolyA-method-SYBR Green | Stem-loop method- TaqMan

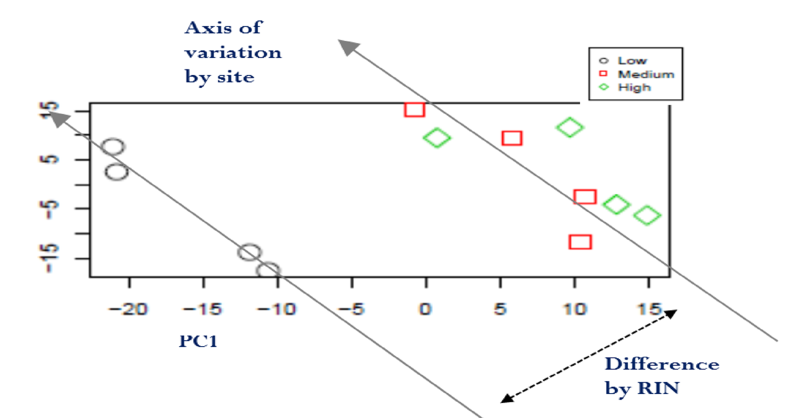
R8
R4
R2

ANALYSIS

Affymetrix miRNA Galaxy GeneChips

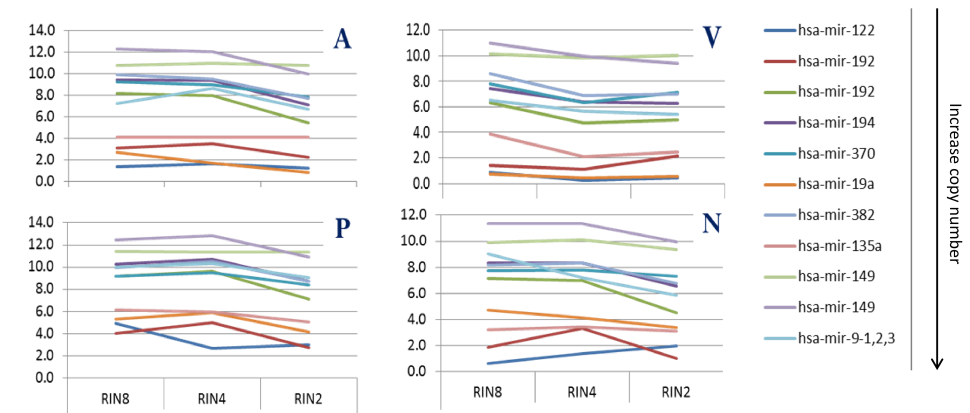
Principal Components Analysis

A clear delineation was observed for both RIN value and site



Microarray Signal for each miRNA Studied

RMA detection (Log_2) of each miRNA by RIN value. Letter denotes lab.



Fold Change Data

RIN 2 RNA has loss of ncRNA as compared to the RIN 8 control.
RIN 4 and 8 show little difference.

FDR < 0.1

Comparison	Fold Change	Un-Normalized	Normalized
RIN 2 vs 8	2 Up	1	11
	2 Down	256	78
RIN 4 vs 8	2 Up	0	0
	2 Down	4	0

For this study the data was not quantile normalized because the resulting effect would be to erroneously enhance the signals of the dim probes sets

CONCLUSIONS

There seems to be a fair amount of inter-lab variability in the qPCR Cq values for miRNA. We believe that this is probably at the level of the RT step as determined previously in the mRNA qPCR studies. Increased degradation is directly proportional to Cq regardless of method. There was not a significant difference between R4 and R8 indicating that some degradation is tolerated. The discrimination between R2 and R4/R8 was better using the MegaPlex stem-loop method.

hsa-miR-122 was only detected by 2 labs (and only with R8 and R4) using the MegaPlex method. This miRNA was however, detected in human brain reference RNA previously (Ambion TechNotes, 2007, 14:2) using the MegaPlex method with pre-amplification step. This miRNA was detected by all labs using the polyA tailing method suggesting a lower detection threshold.

The polyA tailing method tended to give more false positives for the hsa-miR-370 assay.

There was little difference in Cq value between R8, R4 and R2 using the stem-loop method for hsa-miR-370.

Microarray is a very robust method at detecting miRNA's. However as RNA is degraded, a significant number of miRNAs (256) showed reduced detection by 2 fold or higher. We caution users on using normalization methods while analyzing miRNA microarray data.

Microarray data for R8 and R4 cluster very closely together and have very similar miRNA detection profiles, while R2 had significantly reduced signal. Microarray data mimics the RT-qPCR data with respect to copy number trends, clustering, and overall ability to detect miRNA.

Based on our data, it is apparent that total RNA with RIN values below 5 be handled cautiously as resulting data may be compromised as well. More importantly it is not encouraged to compare data from two RNA's with drastically different qualities (ie R2 vs R8).

ACKNOWLEDGEMENTS

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