A novel and universal method for microRNA RT-qPCR data normalization

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outline

- megaplex stem-loop RT-PCR & PreAmp preamplification
- normalization of microRNA gene expression levels

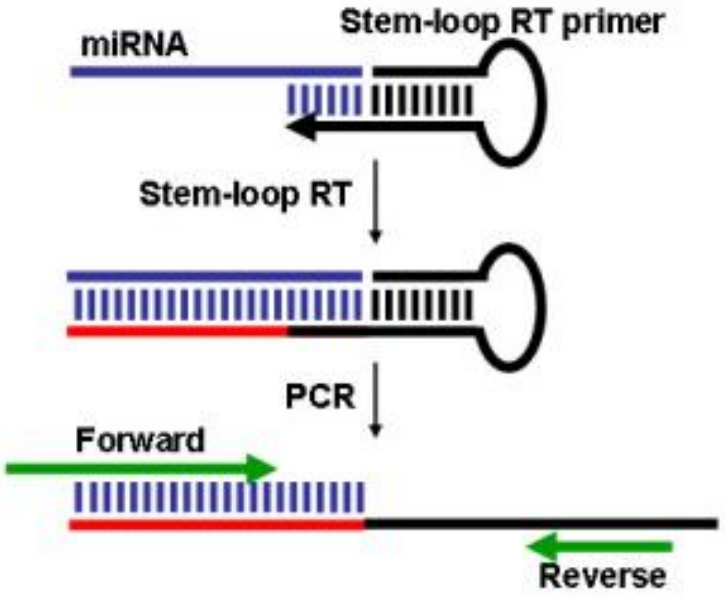
microRNA quantification platform

- hybridisation based (microarray or beads in solution)
  - Exiqon probeset | miRCURY arrays | flexmiR beads
  - Ambion mirVana probeset
  - Invitrogen NCode probeset
  - Agilent Human miRNA Microarray
  - Asuragen DiscovArray (service)
- PCR based
  - Applied Biosystems stem-loop RT-PCR
  - Exiqon miRCURY LNA microRNA PCR System
  - Invitrogen NCodemiRNA RT-PCR
  - Qiagen miScript primer set
  - miQPCR and other home brew protocols
- sequencing based (RNAseq)

microRNA expression profiling

- stem-loop megaplex reverse transcription using 20 ng total RNA
- limited-cycle pre-amplification
- qPCR profiling 450 miRNAs and controls

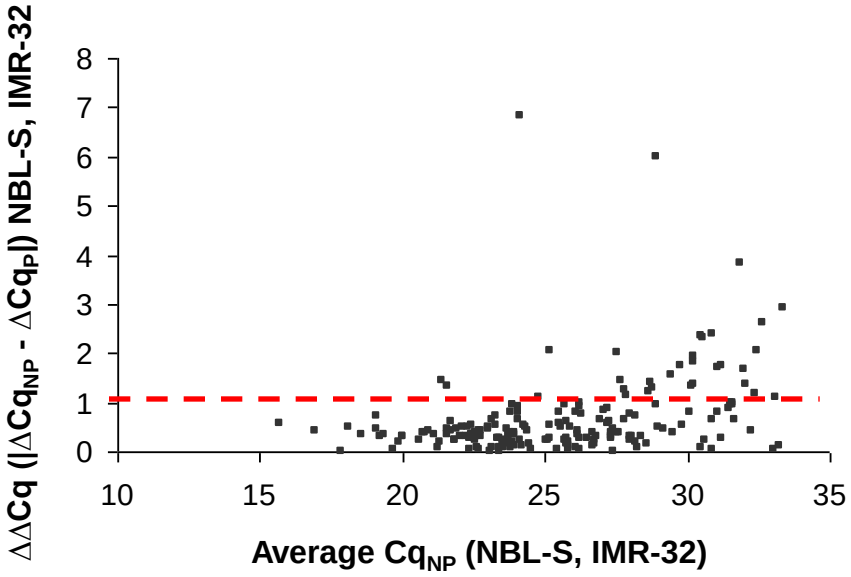
- higher sensitivity
- minimal amplification bias (Mestdagh et al., Nucleic Acids Research, 2008)
- profiled > 1000 samples

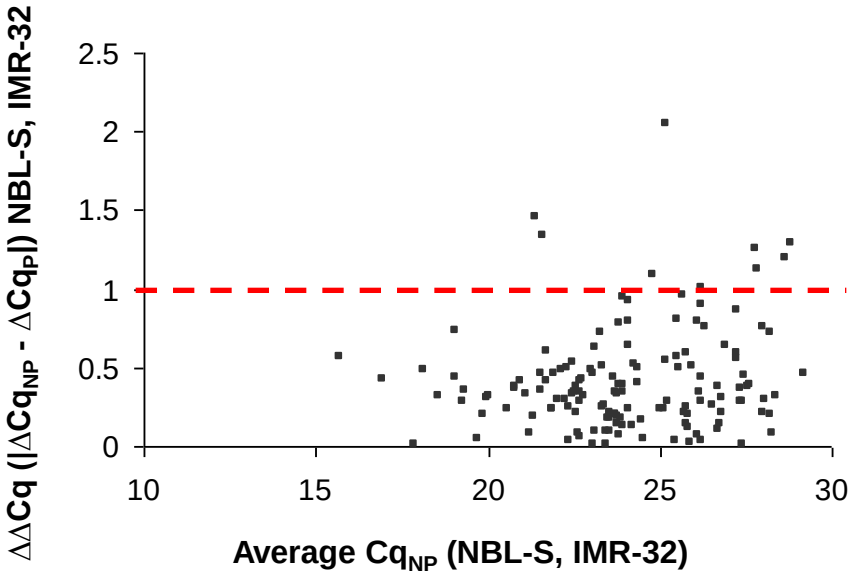


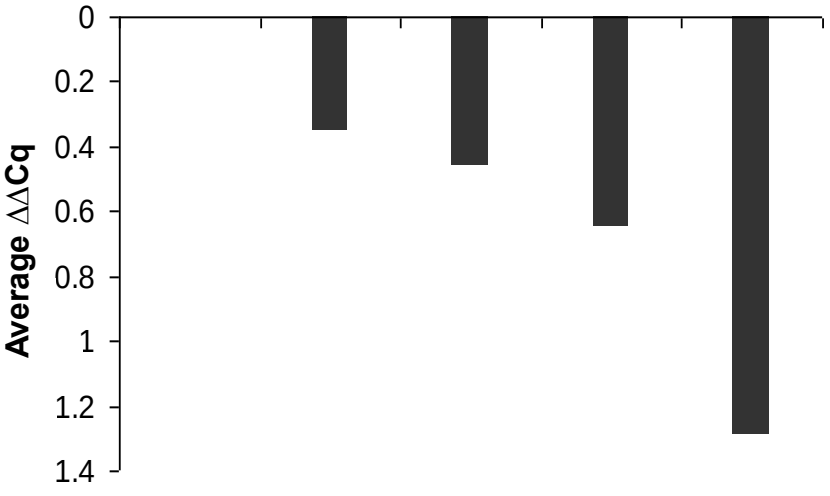
platform evaluation

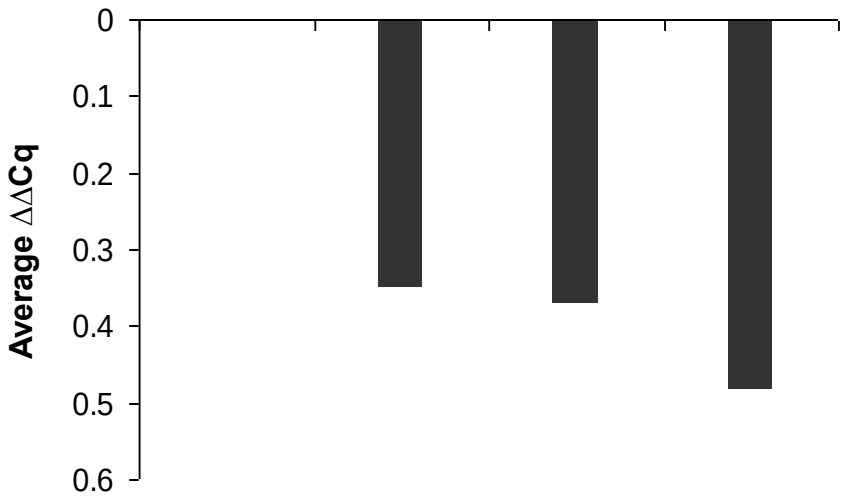
- RT-qPCR
  - qPCR plate setup: gene maximization (Hellemans et al., Genome Biology, 2007)
    - *a different miRNA in each well of a 384 well plate (no replicates)*
    - *1 sample per 384 well plate*
  - sample input
    - *20 ng total RNA (PreAmp)*
    - *1.6 µg total RNA*
  - liquid handling Tecan Evo
  - qPCR reactions on 7900HT
  - quality control
    - *mean Cq for each 384 well plate*
    - *number of not expressed miRNAs*

minimal pre-amplification bias

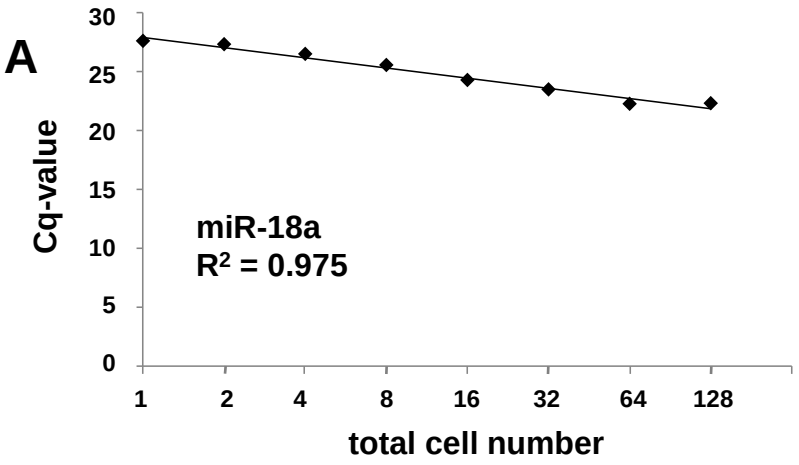


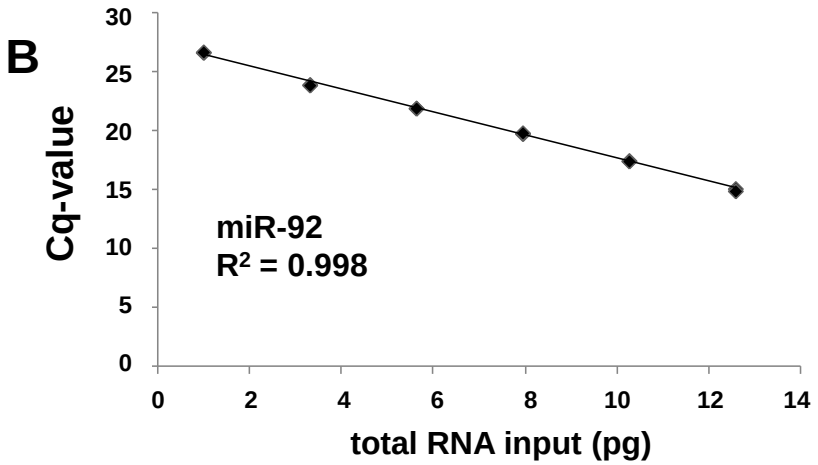


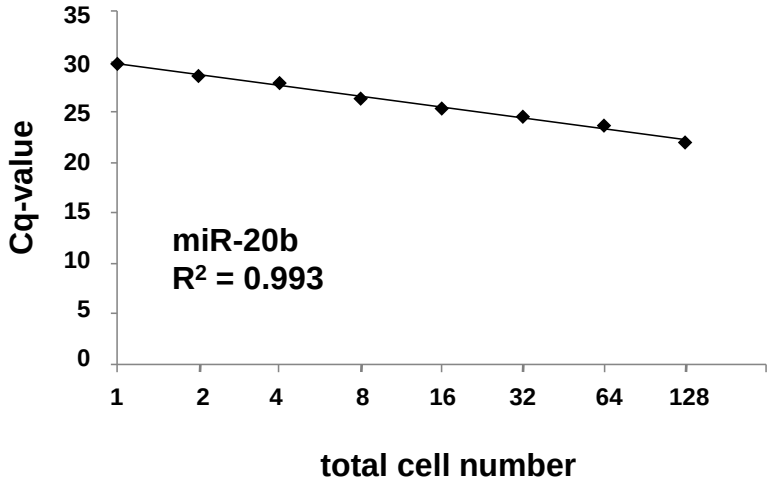


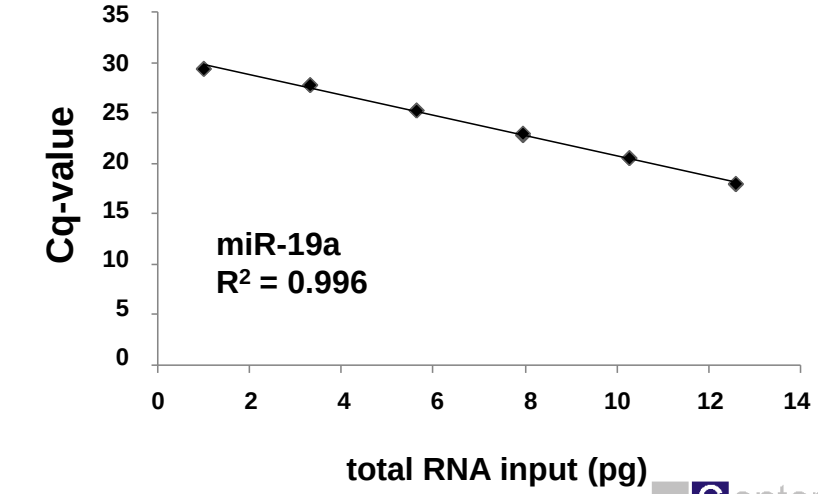


single cell profiling









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High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA

Pieter Mestdagh[1], Tom Feys[1], Nathalie Bernard[2], Simone Guenther[2], Caifu Chen[2], Frank Speleman[1] and Jo Vandesompele[1,*]

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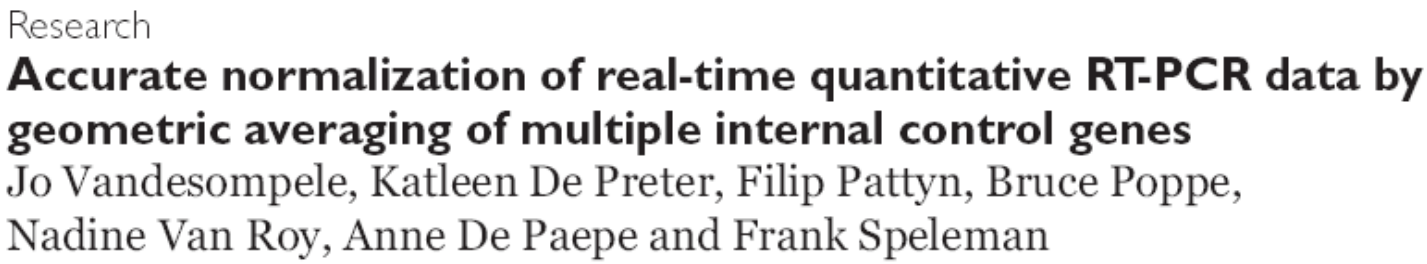
normalization

- removal of experimentally induced noise
  - input quantity: RNA quantity, cDNA synthesis efficiency, …
  - input quality: RNA integrity, RNA purity, …

- gold standard is the use of multiple stably expressed reference genes
  - which genes?
  - how many?
  - how to do the calculations?

geNorm normalisation

- framework for qPCR gene expression normalisation using the reference gene concept:
  - quantified errors related to the use of a single reference gene (> 3 fold in 25% of the cases; > 6 fold in 10% of the cases)
  - developed a robust algorithm for assessment of expression stability of candidate reference genes
  - proposed the geometric mean of at least 3 reference genes for accurate and reliable normalisation
  - Vandesompele et al., Genome Biology, 2002



Research

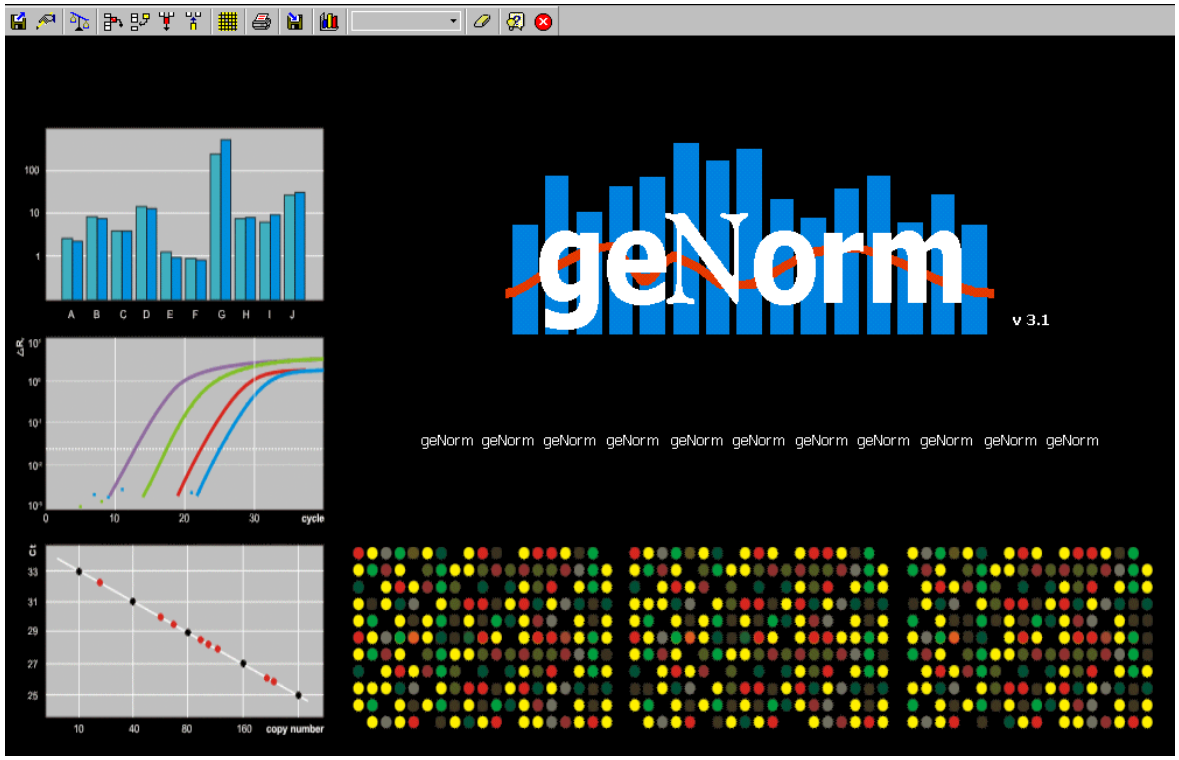
Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes

Jo Vandesompele, Katleen De Preter, Filip Pattyn, Bruce Poppe, Nadine Van Roy, Anne De Paepe and Frank Speleman

  - > 1250 citations in PubMed
  - > 8000 software downloads

geNorm software

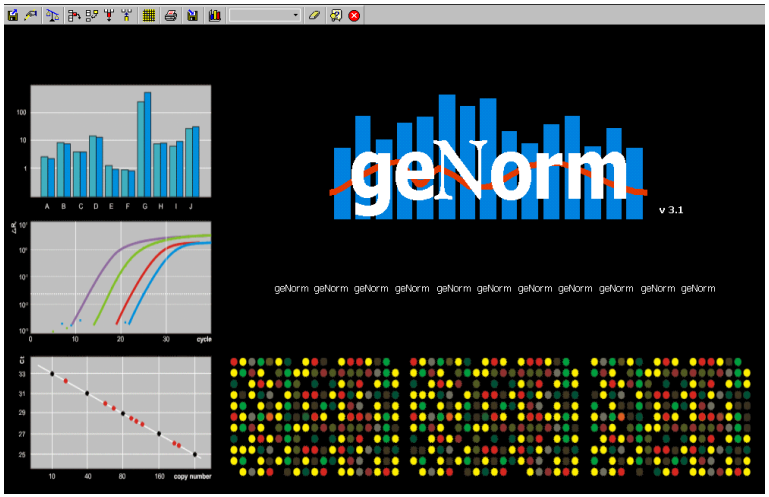
- automated analysis
  - ranking of candidate reference genes according to their stability
  - determination of how many genes are required for reliable normalization



http://medgen.ugent.be/genorm

geNorm validation

- robust – insensitive to outliers
- maximal reduction of experimental variation
- accurate assessment of small expression differences
- statistically more significant results



microRNA normalization

- small-RNA controls
  - classic normalization strategy
  - small nuclear RNAs, small nucleolar RNAs
  - 18 available from Applied Biosystems

- mean normalization
  - method applied for microarray data
  - universal: applicable for every miRNA dataset
  - many datapoints needed (megaplex vs. multiplex)

- miRNAs/controls that resemble the mean
  - minimal standard deviation when comparing miRNA expression with mean (geNorm V value, st dev of log transformed ratios)
  - compatible with multiplex asays
  - need to determine mean

small RNA controls

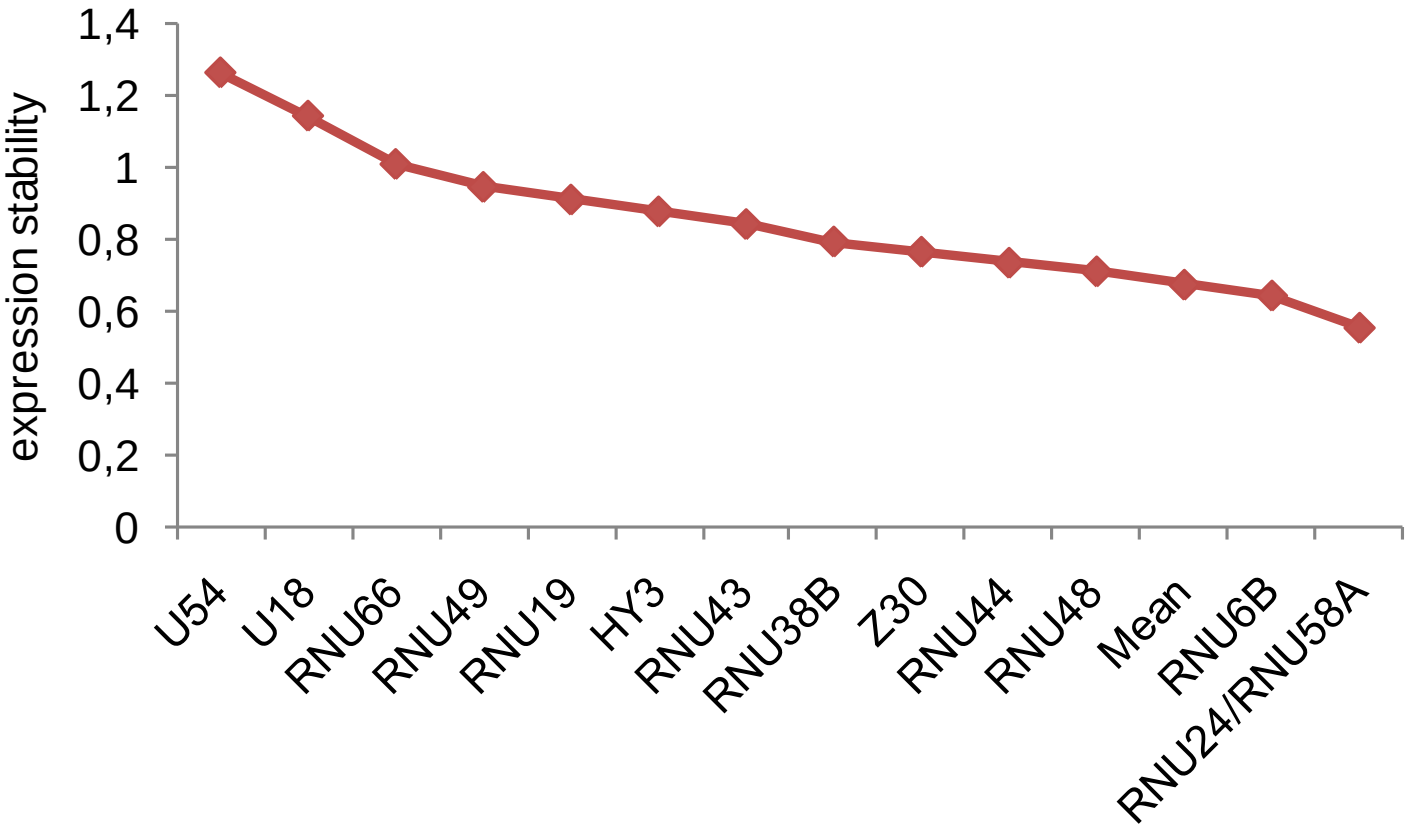
- How ‘stable’ is the mean compared to controls?
  - geNorm analysis using controls and mean as input variables
  - exclusion of potentially co-regulated controls

HY3	7q36
RNU19	5q31.2
RNU24	9q34
RNU38B	1p34.1-p32
RNU43	22q13
RNU44	1q25.1
RNU48	6p21.32
RNU49	17p11.2
RNU58A	18q21
RNU58B	18q21
RNU66	1p22.1
RNU6B	10p13
U18	15q22
U47	1q25.1
U54	8q12
U75	1q25.1
Z30	17q12
RPL21	13q12.2

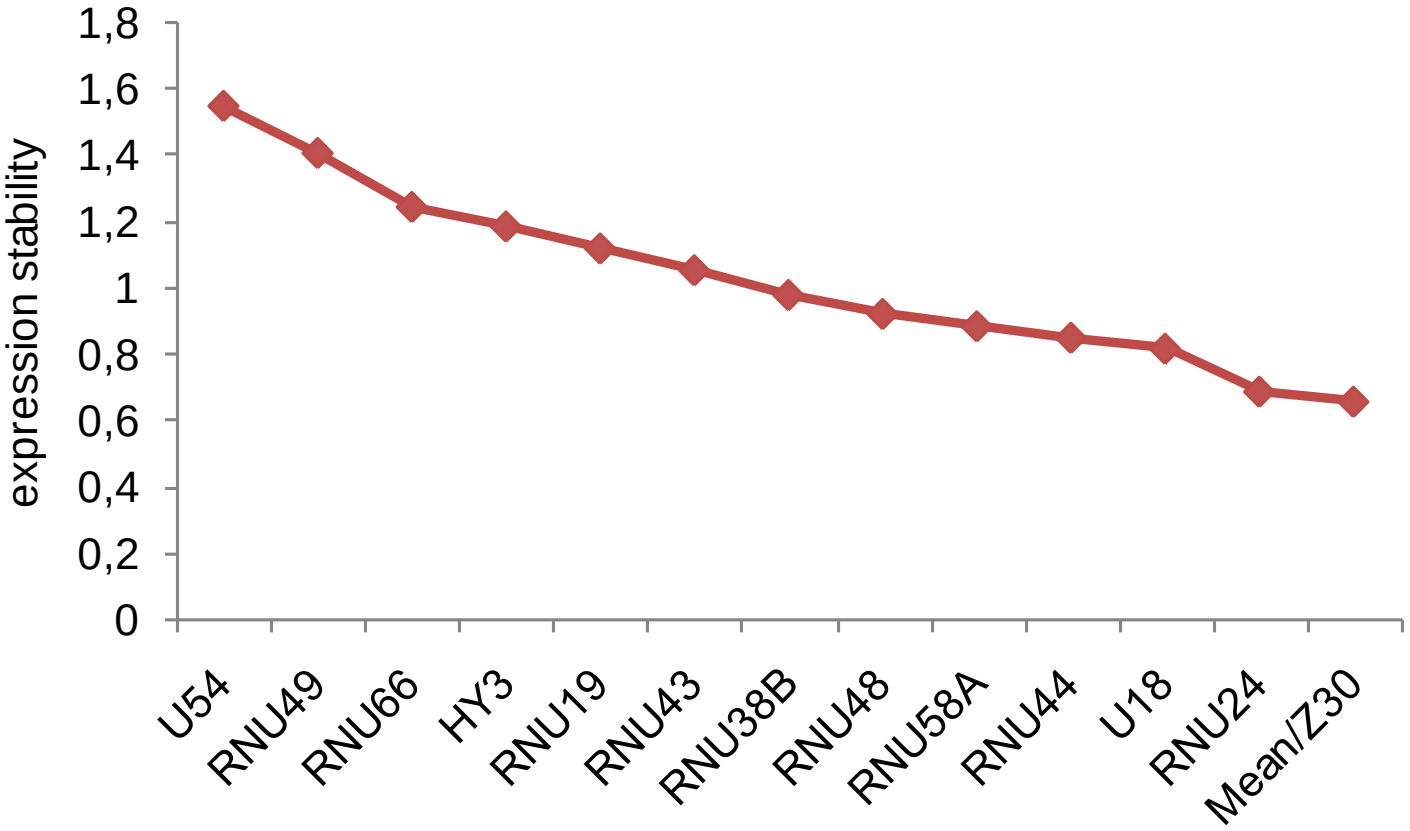
miRNA expression datasets

- neuroblastoma tumour samples
- T-ALL samples
- EVI1 deregulated leukemias
- retinoblastoma tumour samples
- normal tissues
- normal bone marrow

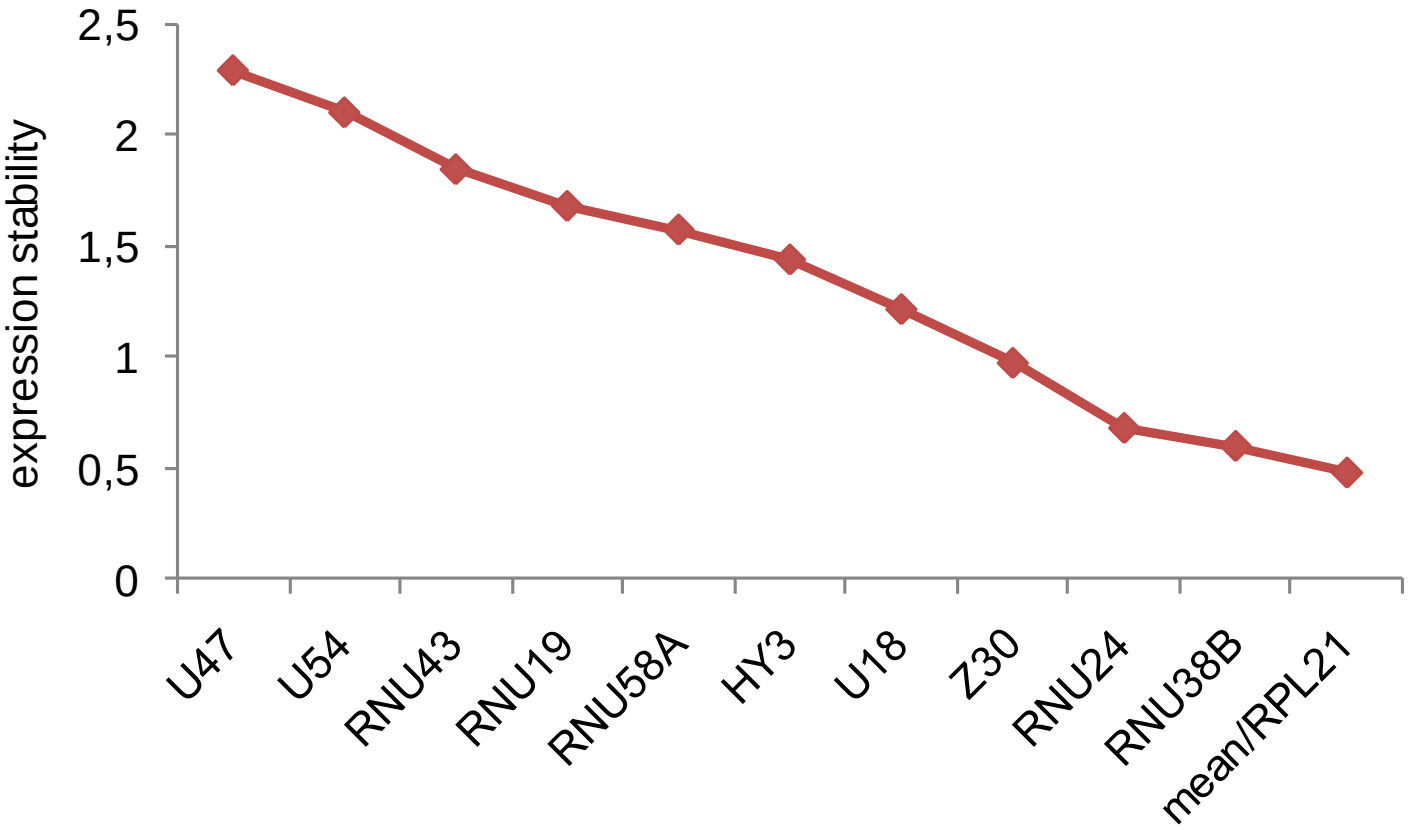
neuroblastoma



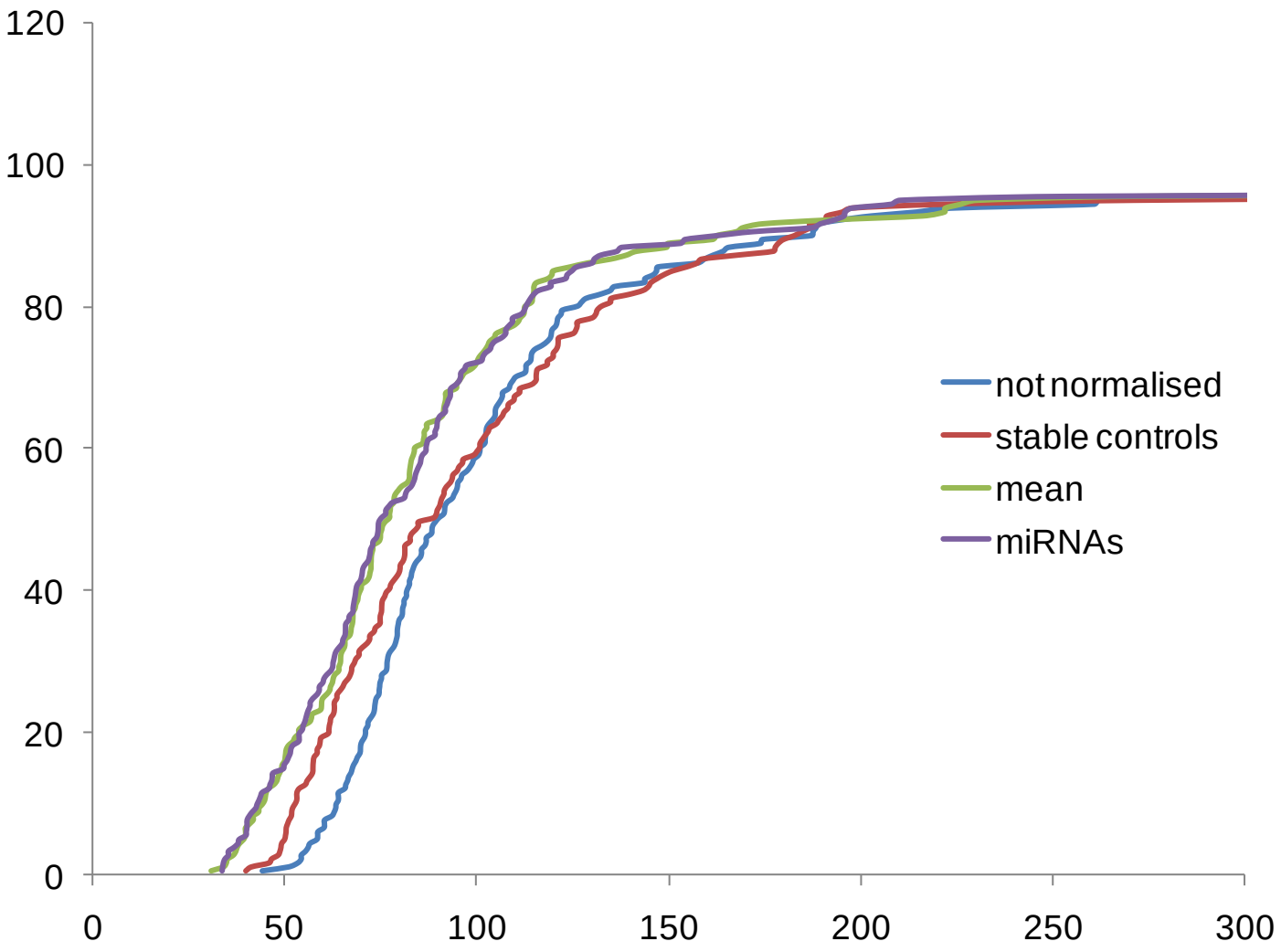
T-ALL


expression stability
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
U54
RNU49
RNU66
HY3
RNU19
RNU43
RNU38B
RNU48
RNU58A
RNU44
U18
RNU24
Mean/Z30

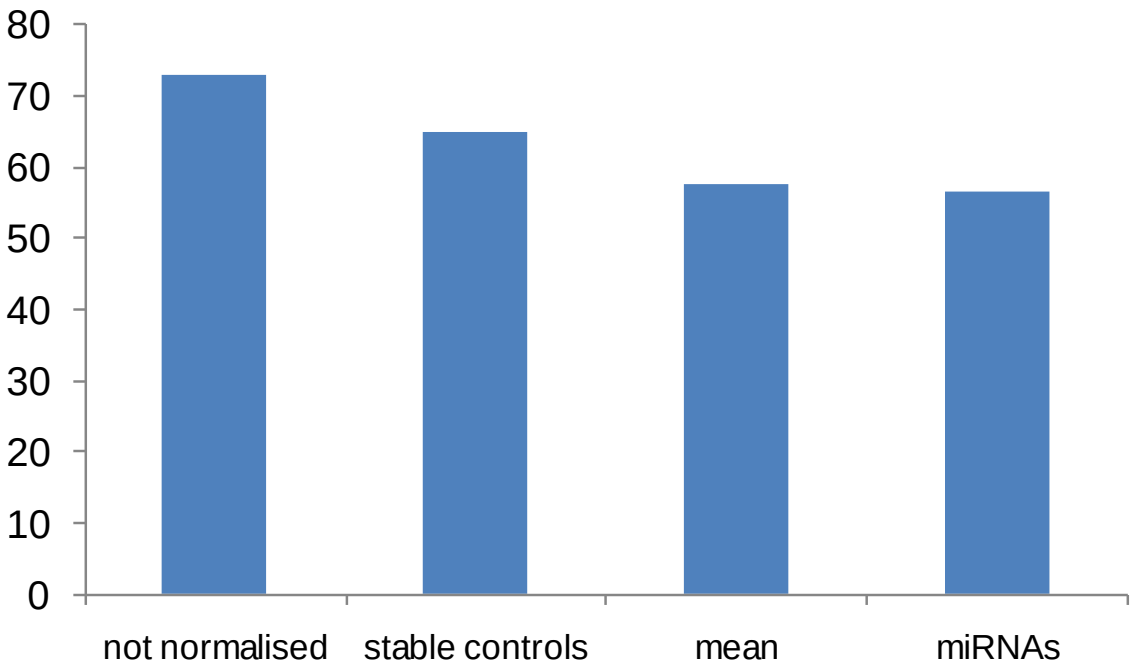
normal tissues



neuroblastoma



neuroblastoma



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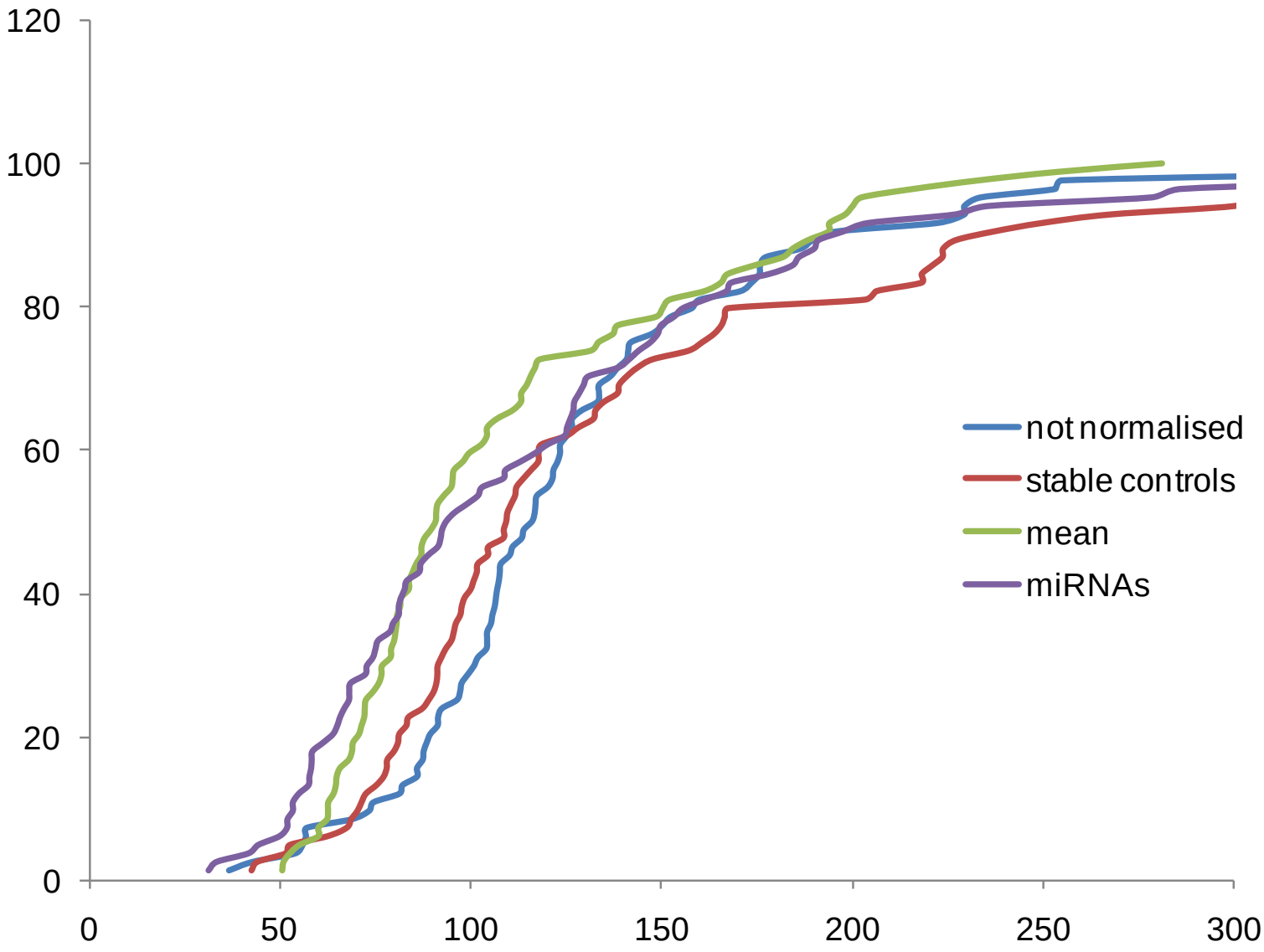
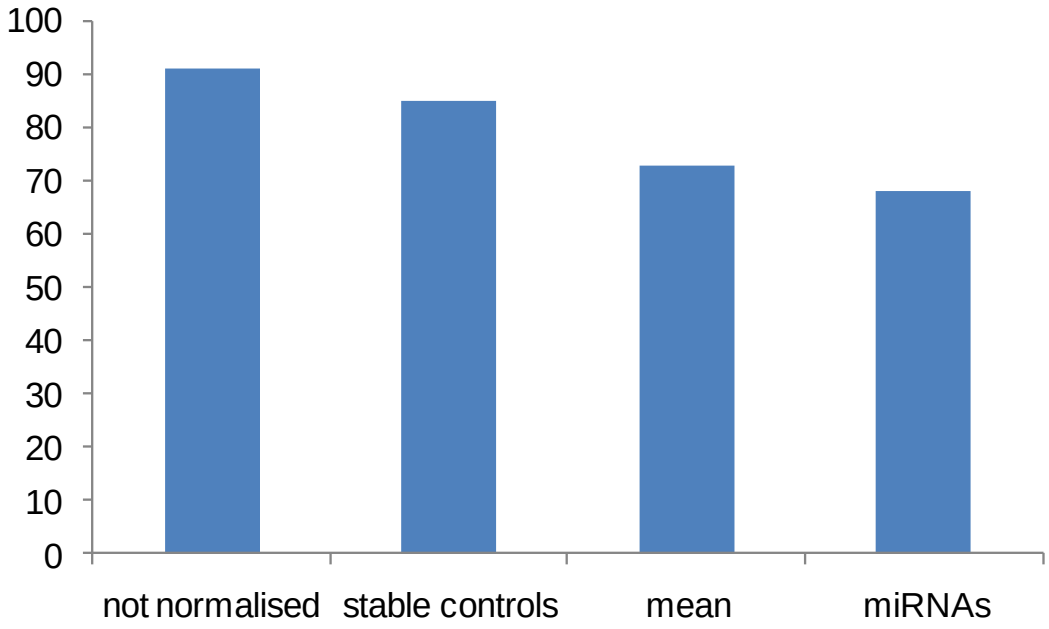
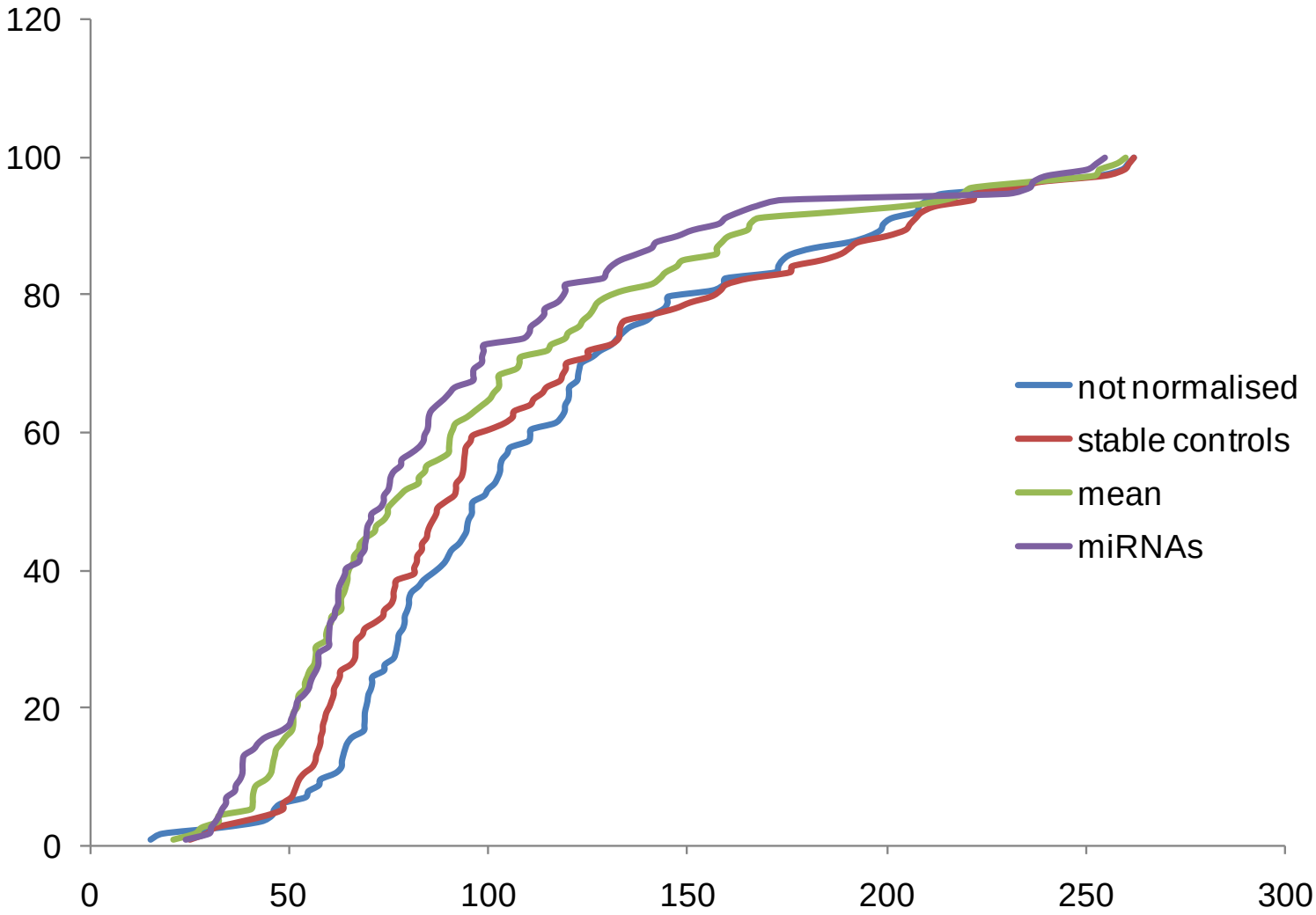

120
100
80
60
40
20
0
0
50
100
150
200
250
300
not normalised
stable controls
mean
miRNAs

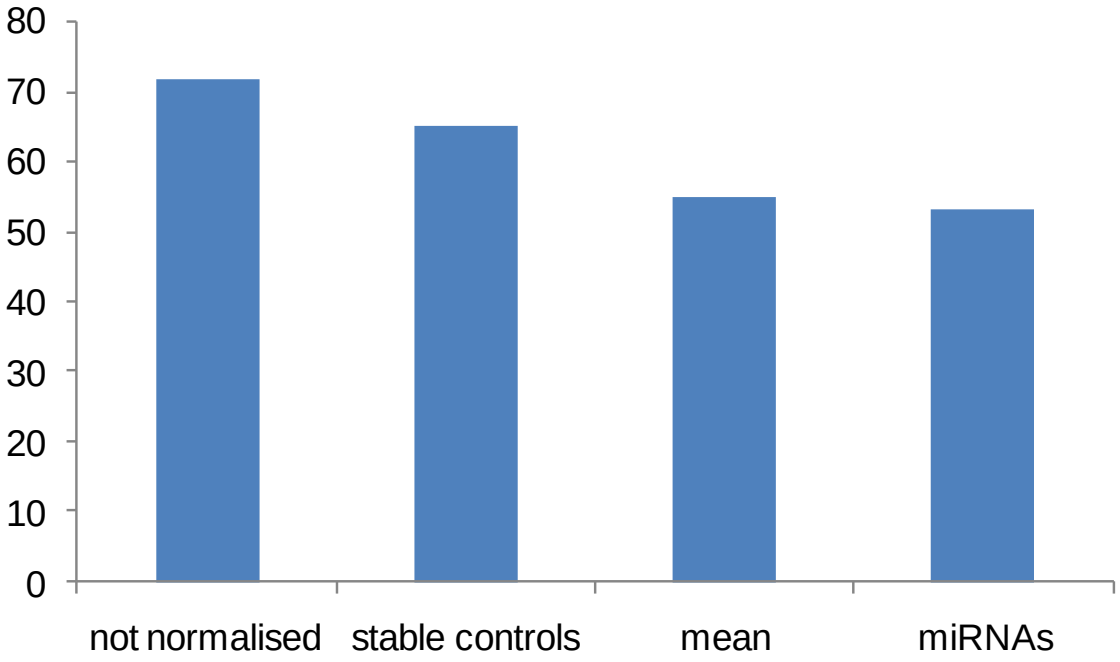
T-ALL


100
90
80
70
60
50
40
30
20
10
0
not normalised
stable controls
mean
miRNAs

normal tissues

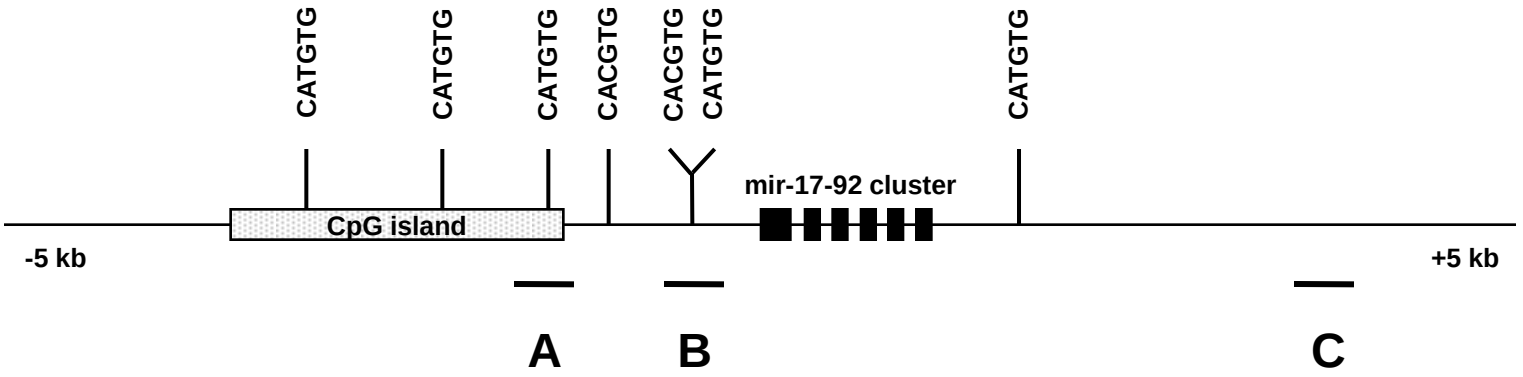


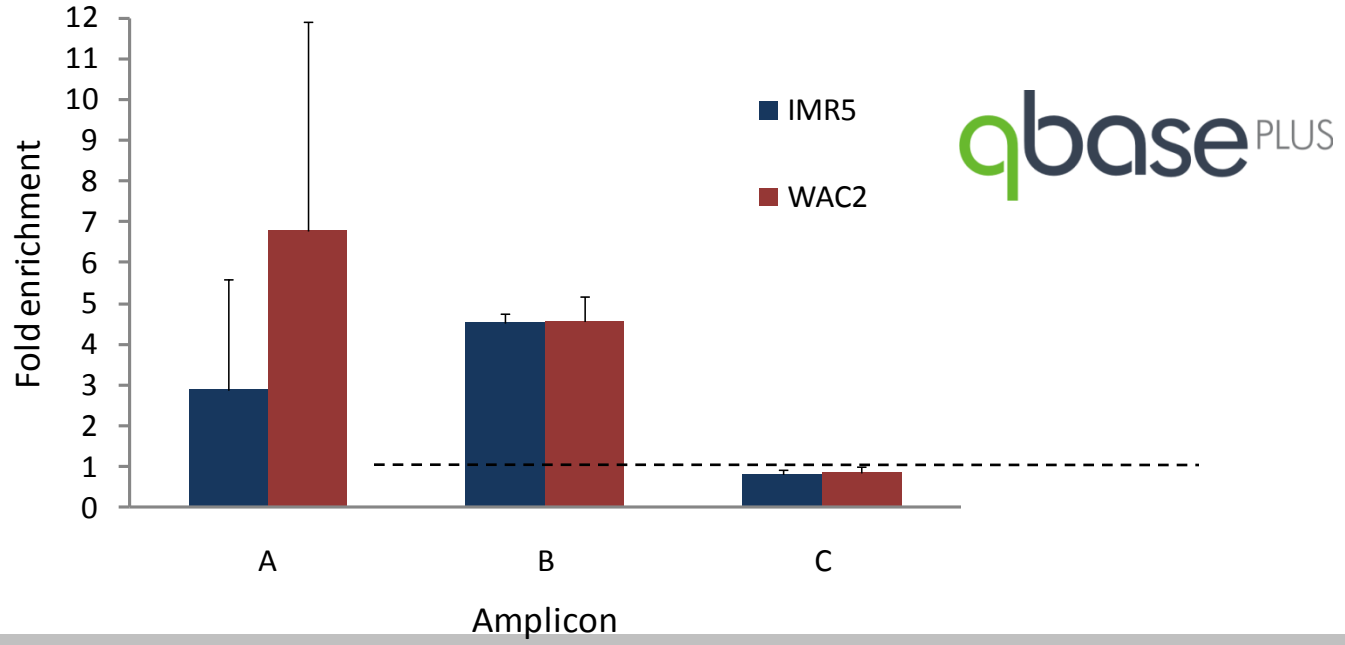
normal tissues



biological validation

- MYCN binds to the mir-17-92 promoter





qbase PLUS

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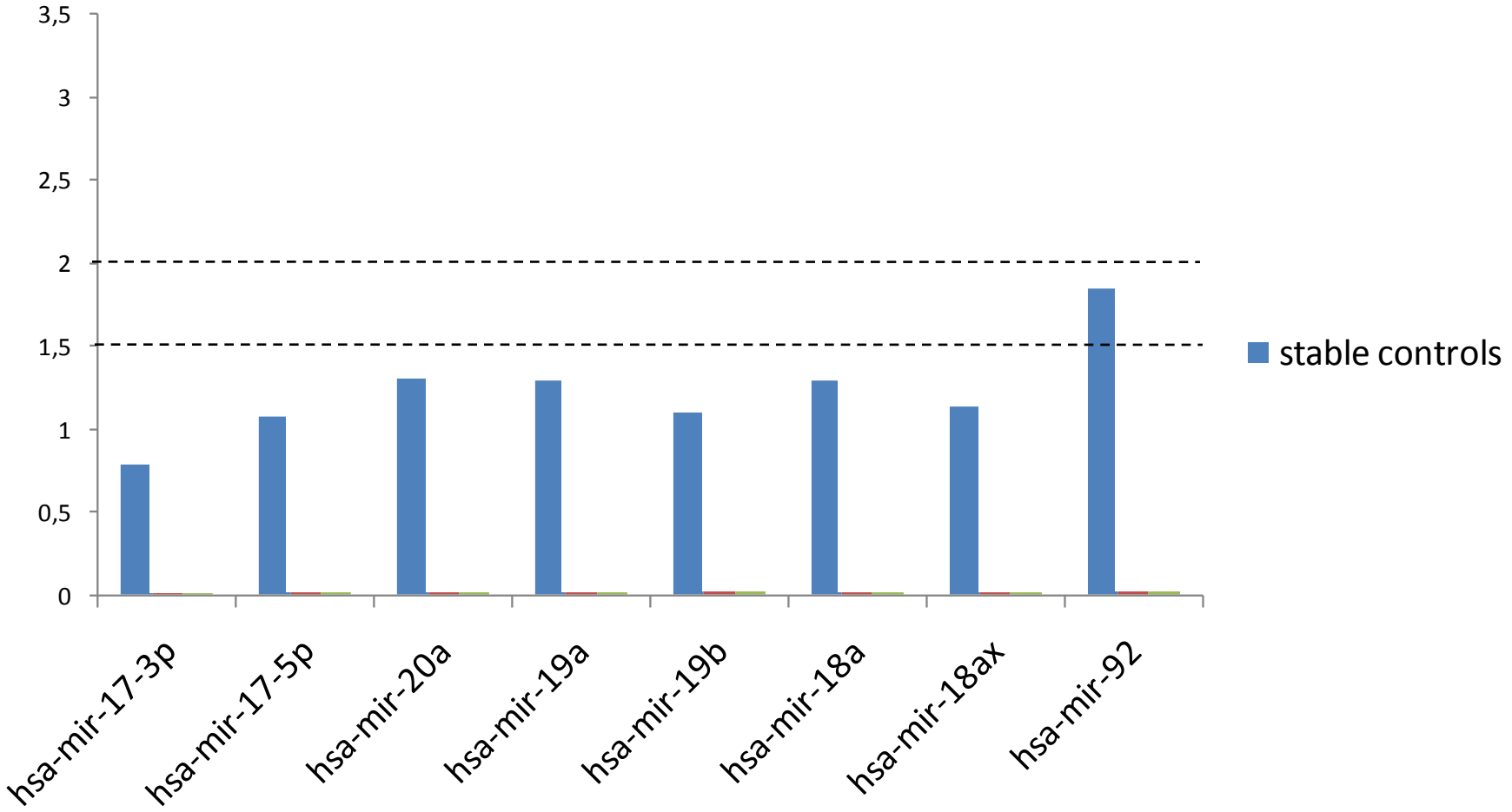
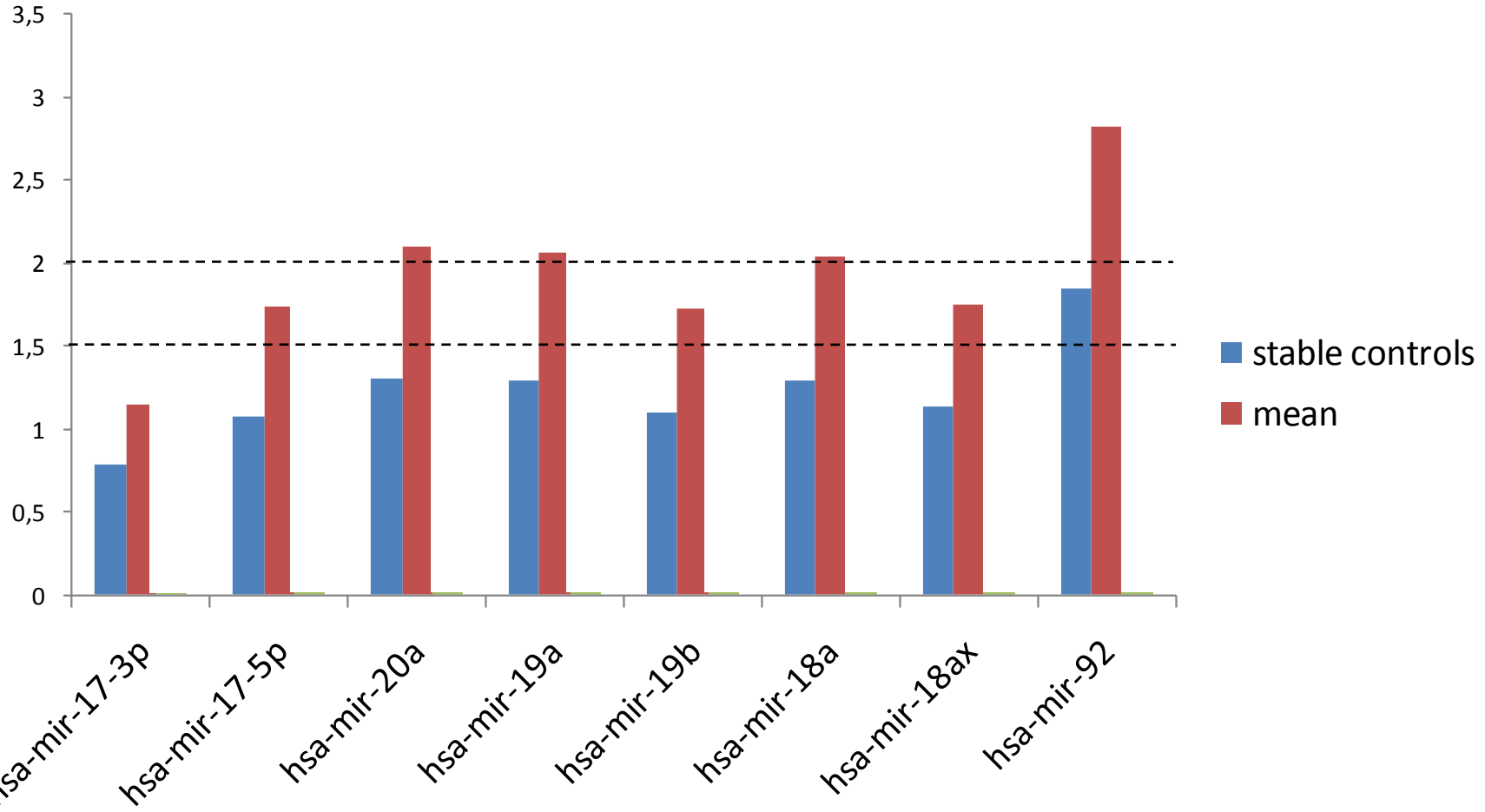
biological validation

choice of normalization strategy influences differential miRNA expression



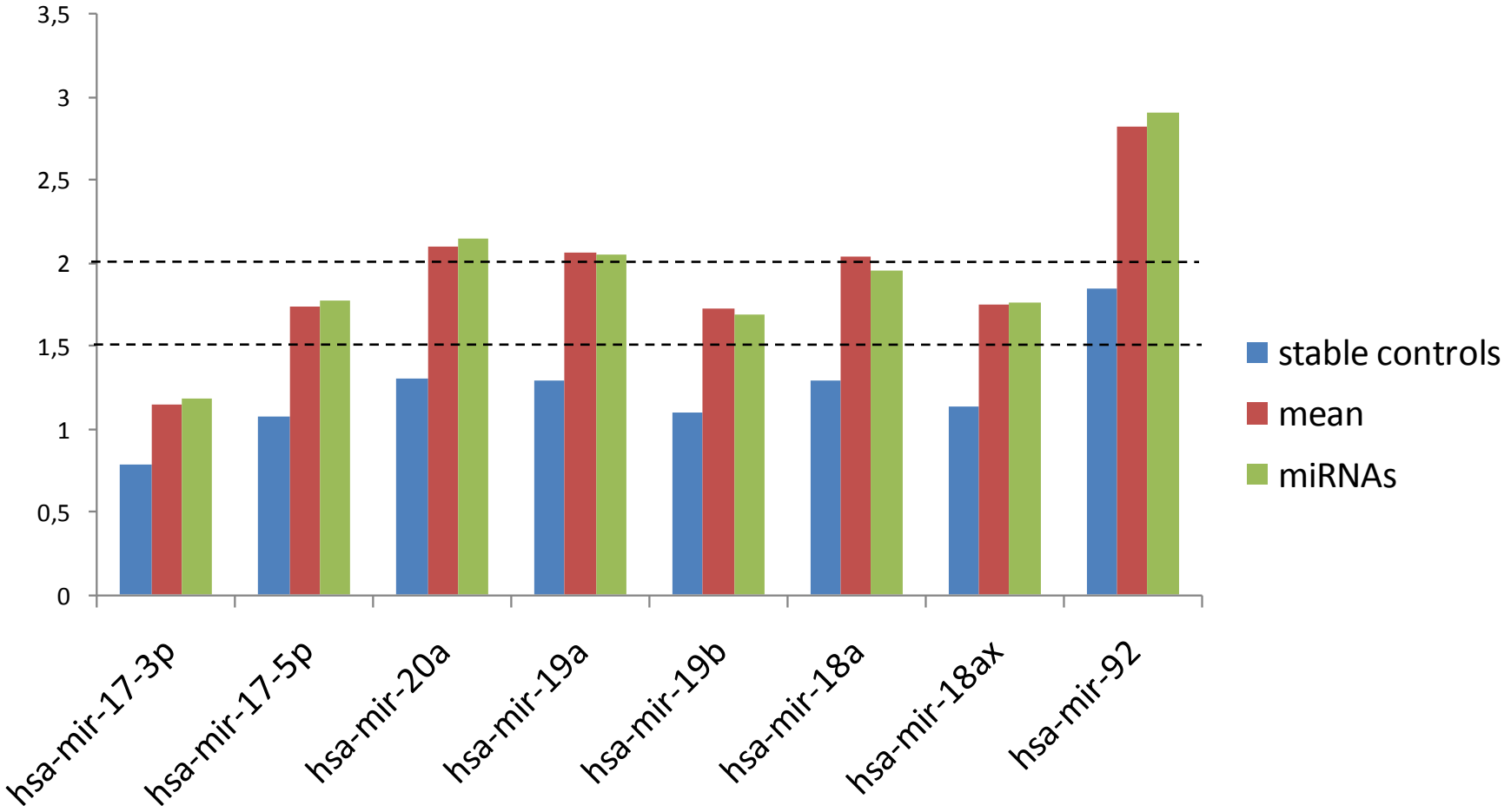
biological validation

- choice of normalization strategy influences differential miRNA expression

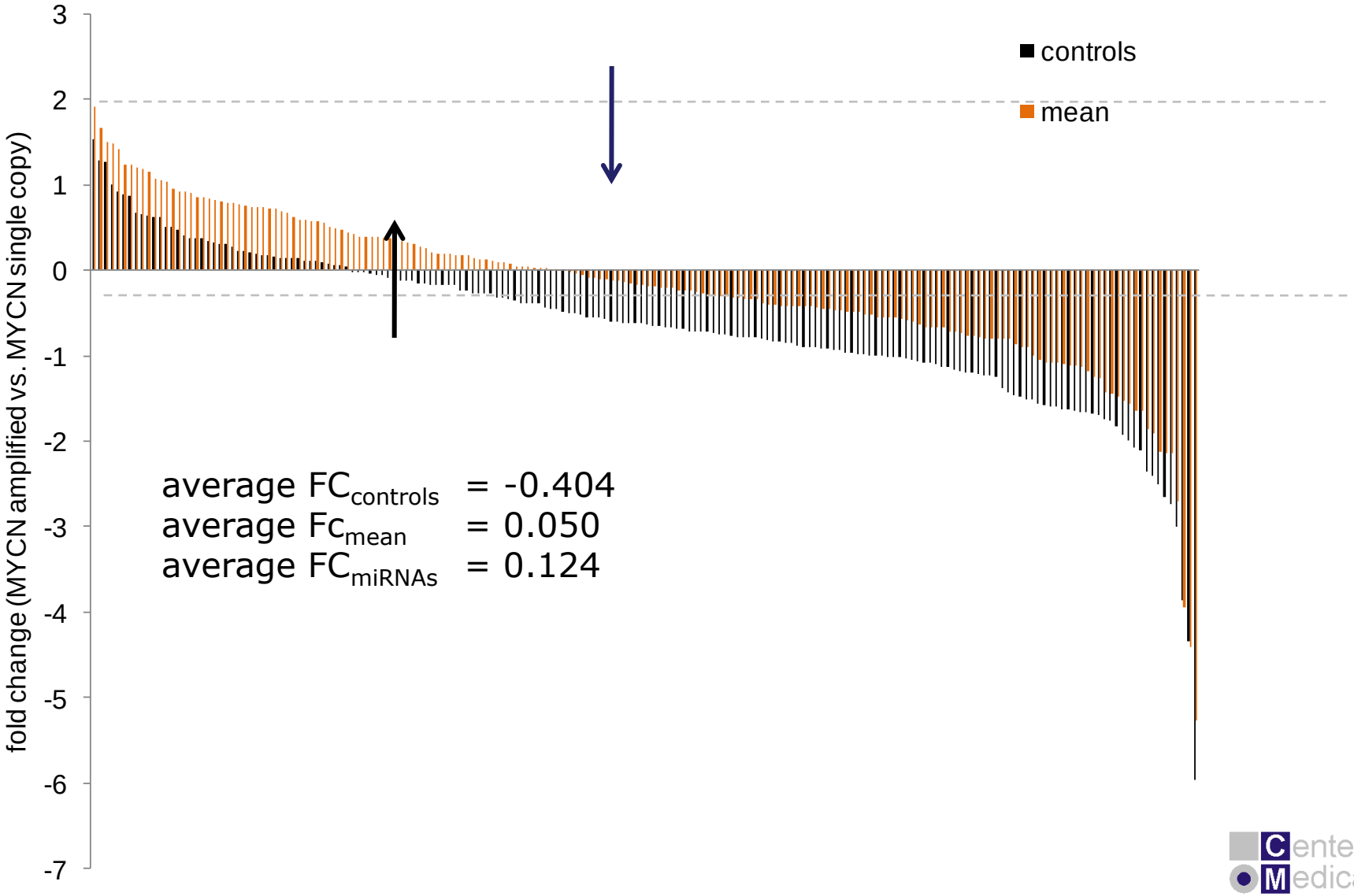


biological validation

- choice of normalization strategy influences differential miRNA expression



balanced differential expression



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conclusions

- a highly sensitive miRNA expression profiling platform

- novel and powerful miRNA normalization strategy
  - maximal reduction of technical noise
  - improved identification of differentially expressed genes
  - balancing of differential expression
  - universally applicable
    - *mean*
    - *multiple stable endogenous controls*

acknowledgments

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