



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

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4<sup>th</sup> International qPCR Symposium  
Weihenstephan, March 10, 2009

# outline

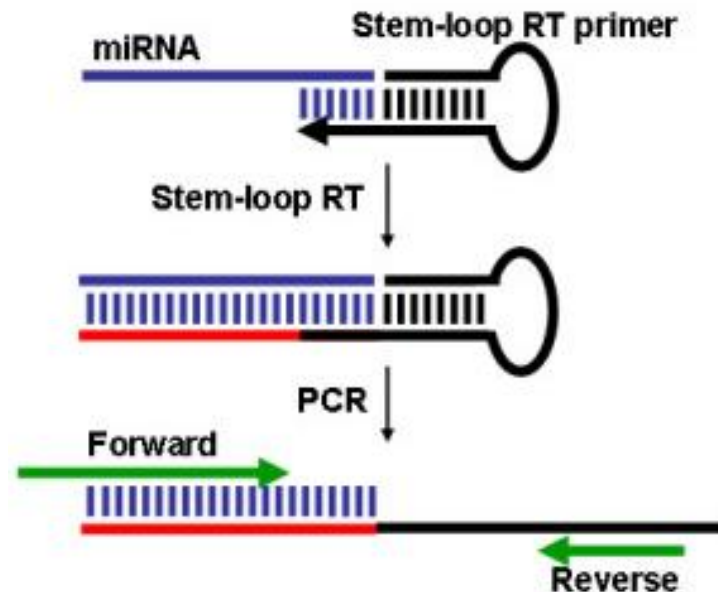
- megaplex stem-loop RT-PCR & PreAmp preamplification
- normalization of microRNA gene expression levels

# 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

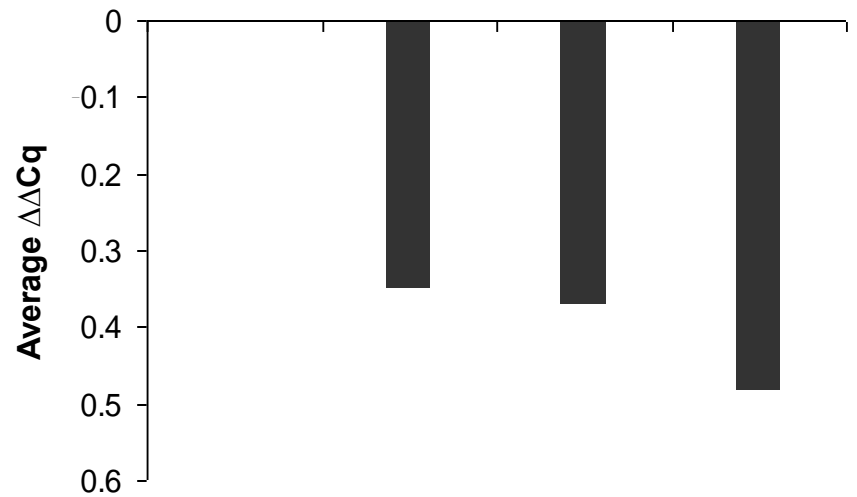
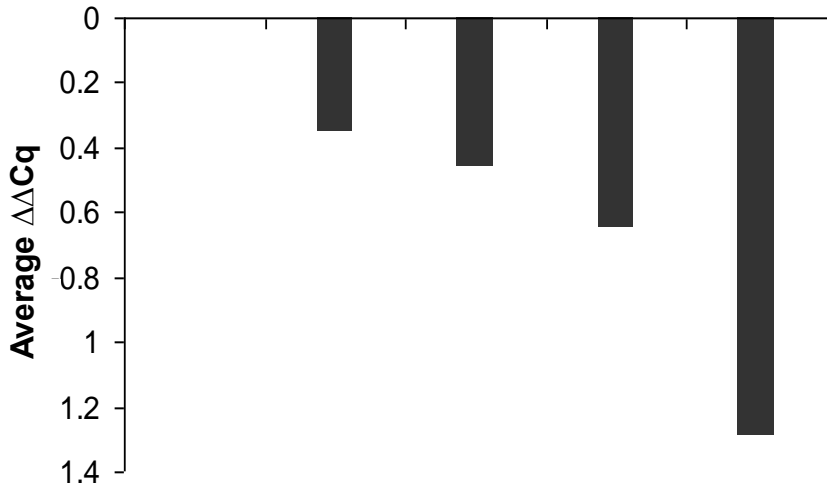
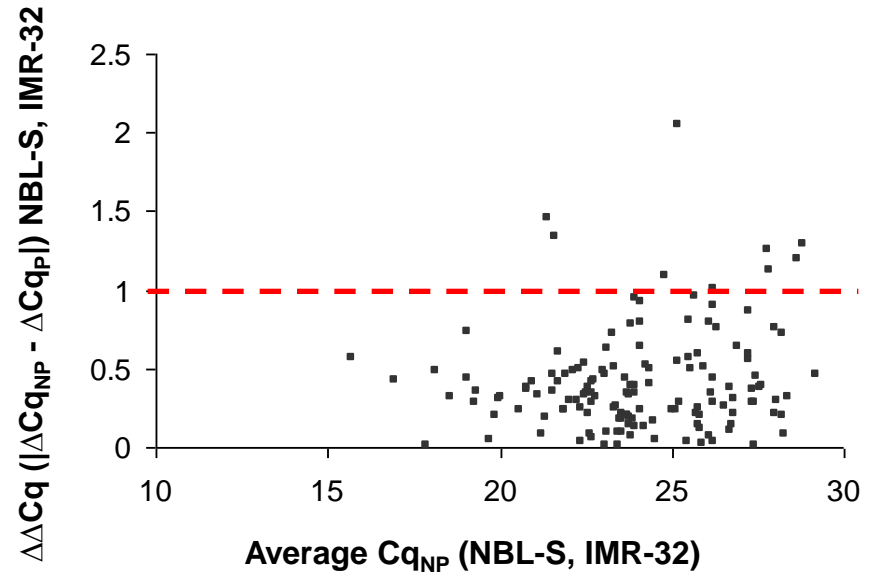
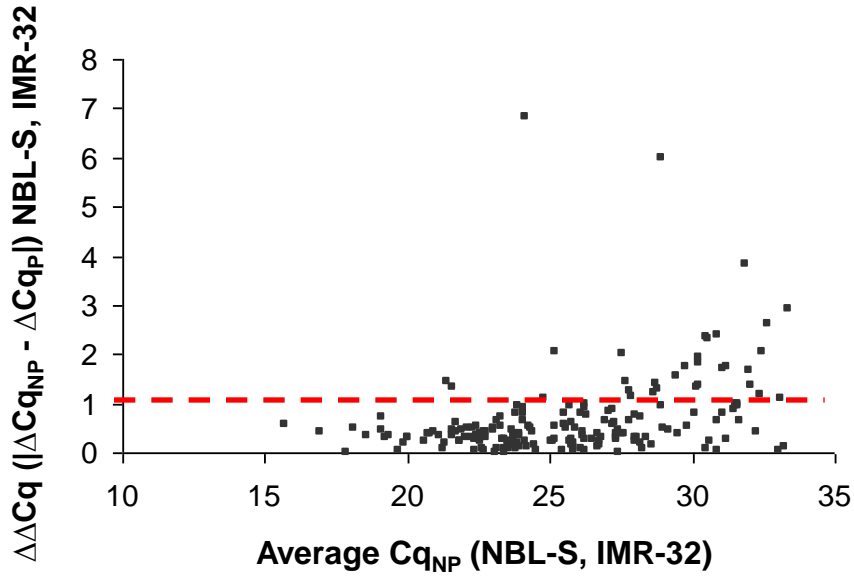


## ■ RT-qPCR

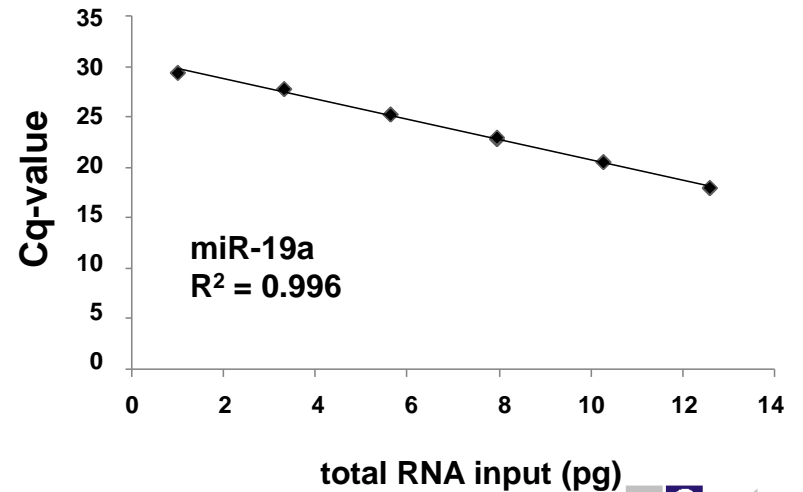
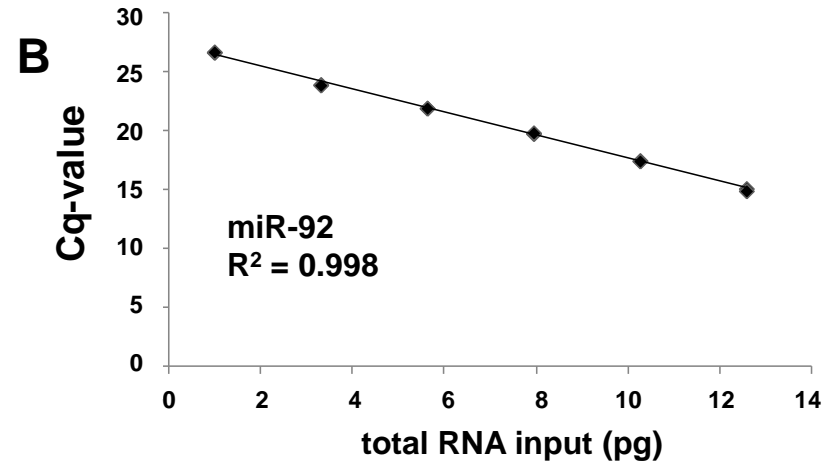
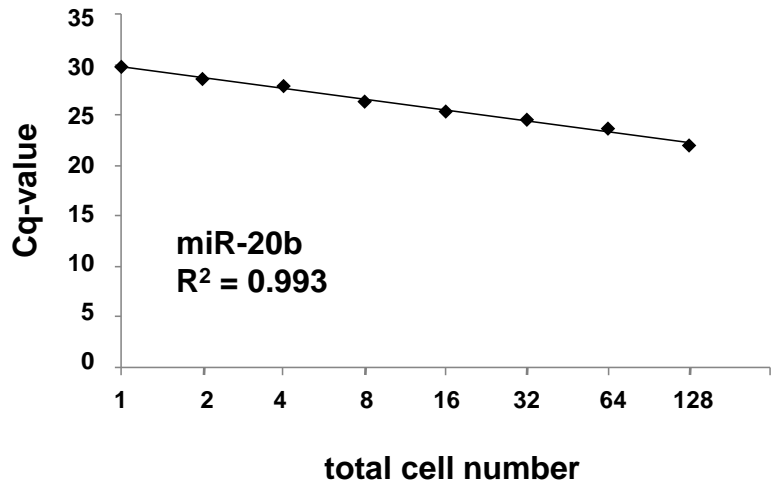
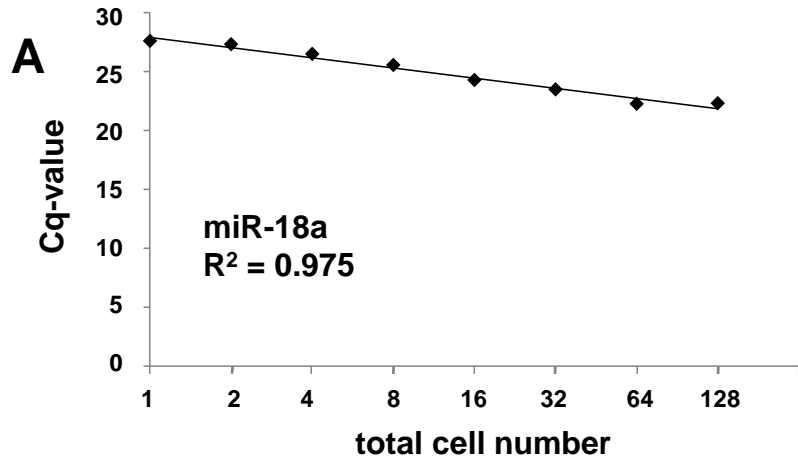
- qPCR plate setup: gene maximization (Hellemans et al., Genome Biology, 2007)
  - o *a different miRNA in each well of a 384 well plate (no replicates)*
  - o *1 sample per 384 well plate*
- sample input
  - o *20 ng total RNA (PreAmp)*
  - o *1.6  $\mu$ g total RNA*
- liquid handling Tecan Evo
- qPCR reactions on 7900HT
- quality control
  - o *mean C<sub>q</sub> for each 384 well plate*
  - o *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<sup>1</sup>, Tom Feys<sup>1</sup>, Nathalie Bernard<sup>2</sup>, Simone Guenther<sup>2</sup>, Caifu Chen<sup>2</sup>, Frank Speleman<sup>1</sup> and Jo Vandesompele<sup>1,\*</sup>

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- 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?

- 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

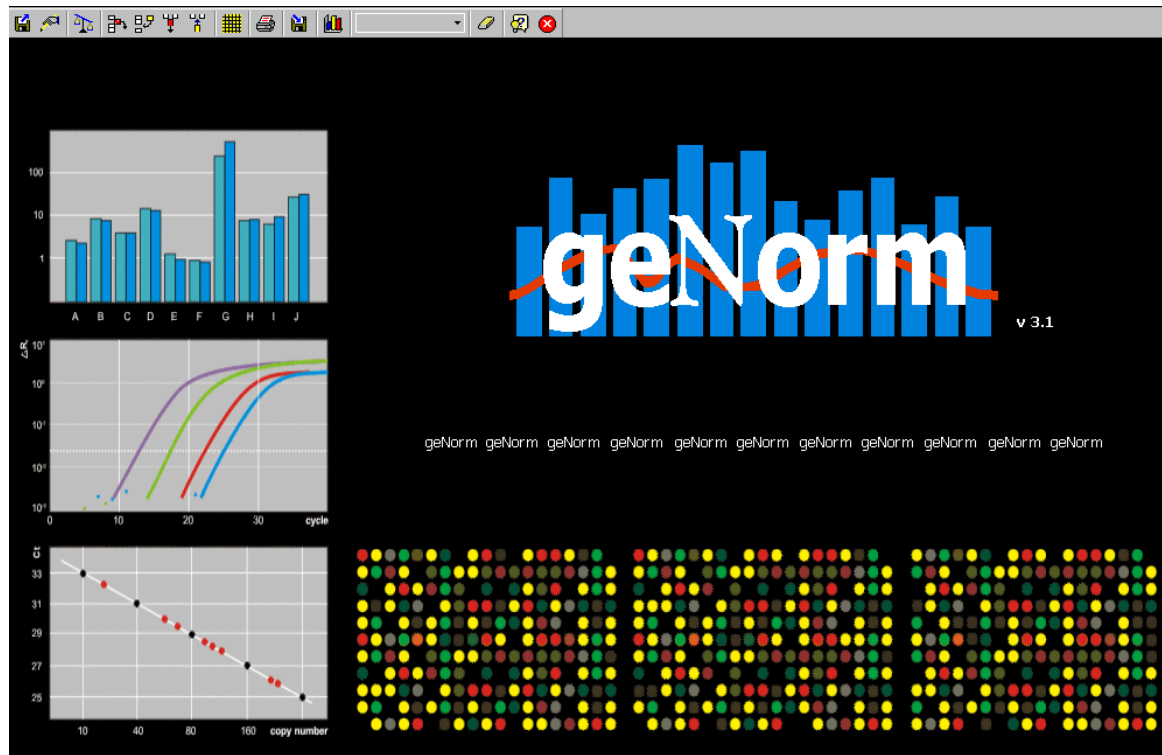
Research

## **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

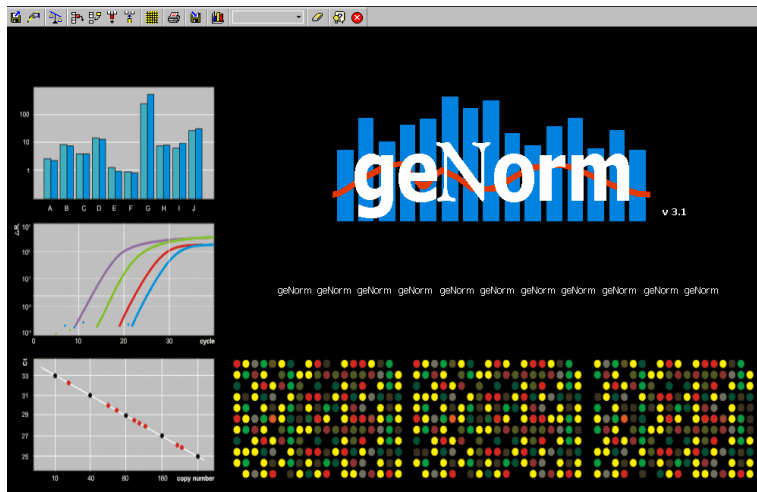
- automated analysis
  - ranking of candidate reference genes according to their stability
  - determination of how many genes are required for reliable normalization



<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



- 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 assays
  - need to determine mean

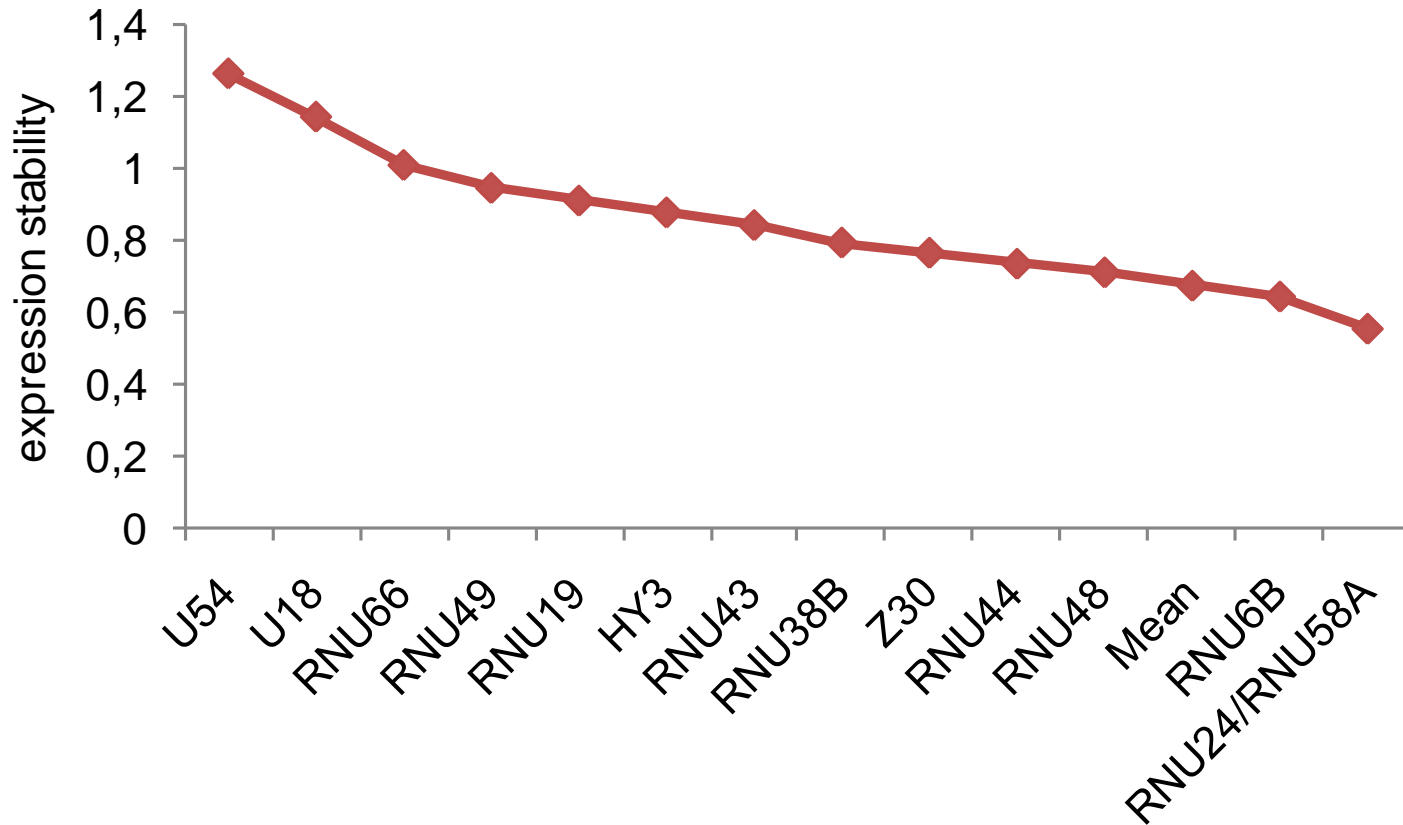
- How 'stable' is the mean compared to controls?
  - geNorm analysis using controls and mean as input variables
  - exclusion of potentially co-regulated controls

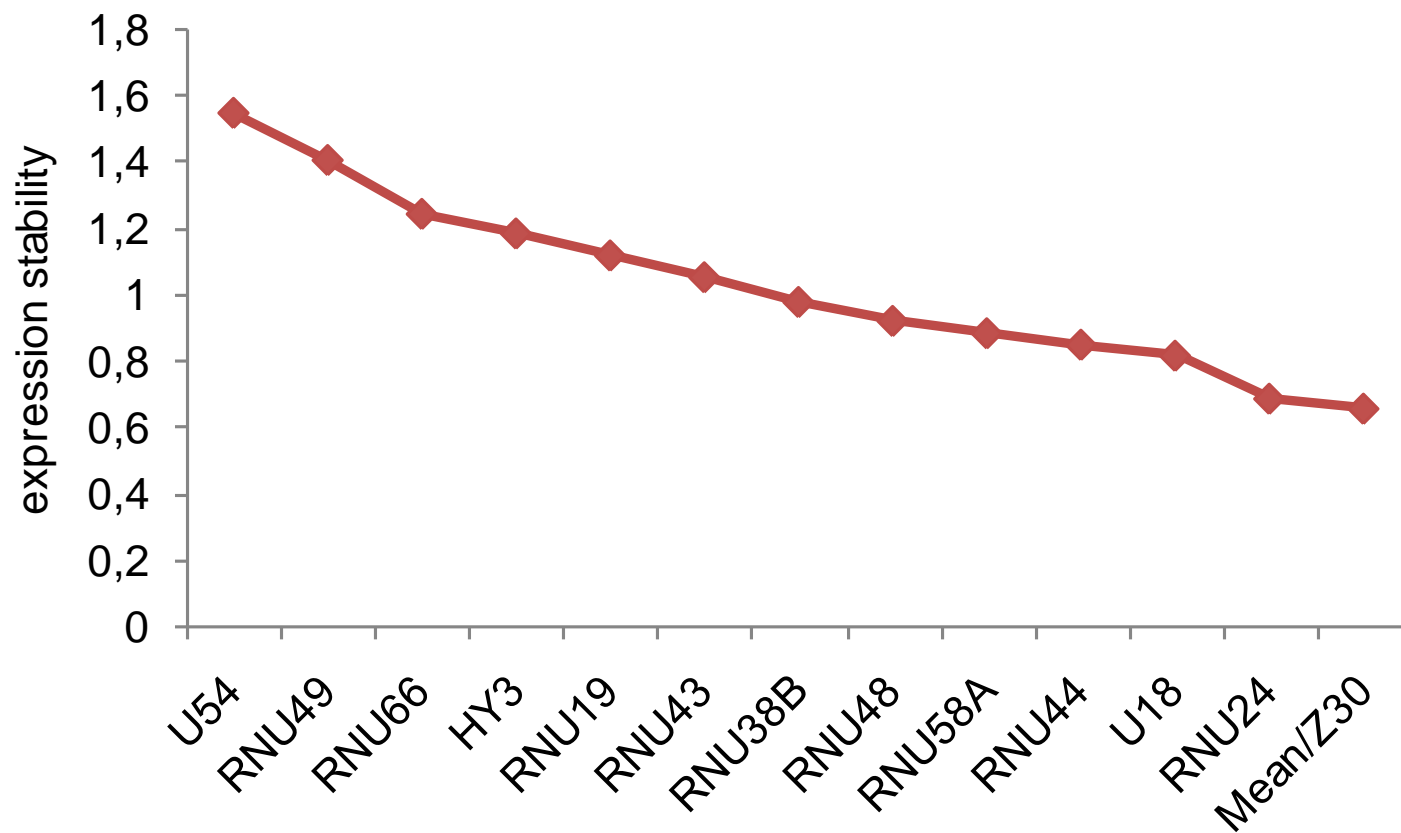
HY3	7q36
RNU19	5q31.2
RNU24	9q34
RNU38B	1p34.1-p32
RNU43	22q13
RNU44	1q25.1
RNU48	6p21.32
RNU49	17p11.2
RNU58A	18q21
RNU58B	18q21
RNU66	1p22.1
RNU6B	10p13
U18	15q22
U47	1q25.1
U54	8q12
U75	1q25.1
Z30	17q12
RPL21	13q12.2

# miRNA expression datasets

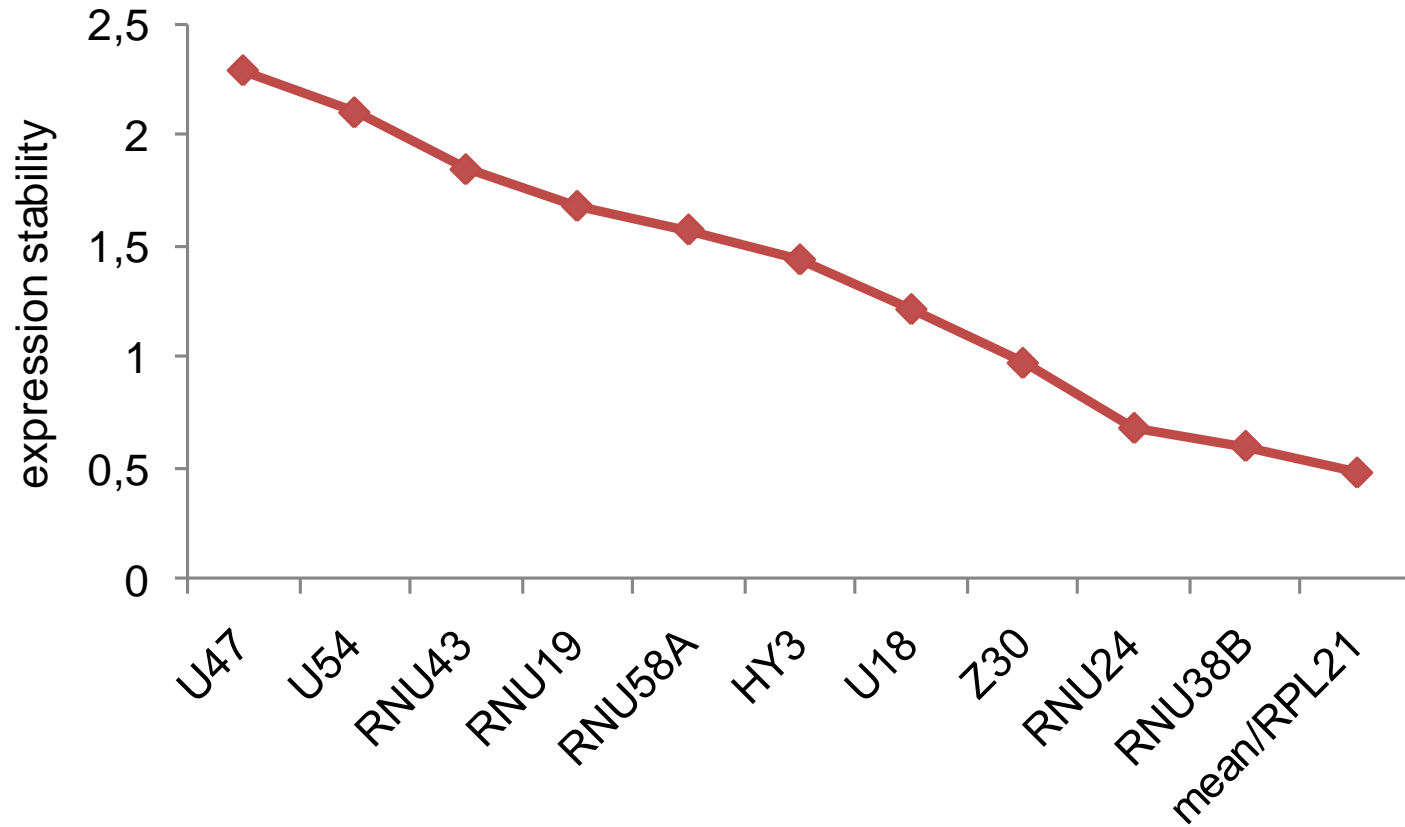
- neuroblastoma tumour samples
- T-ALL samples
- EVI1 deregulated leukemias
- retinoblastoma tumour samples
- normal tissues
- normal bone marrow

# neuroblastoma

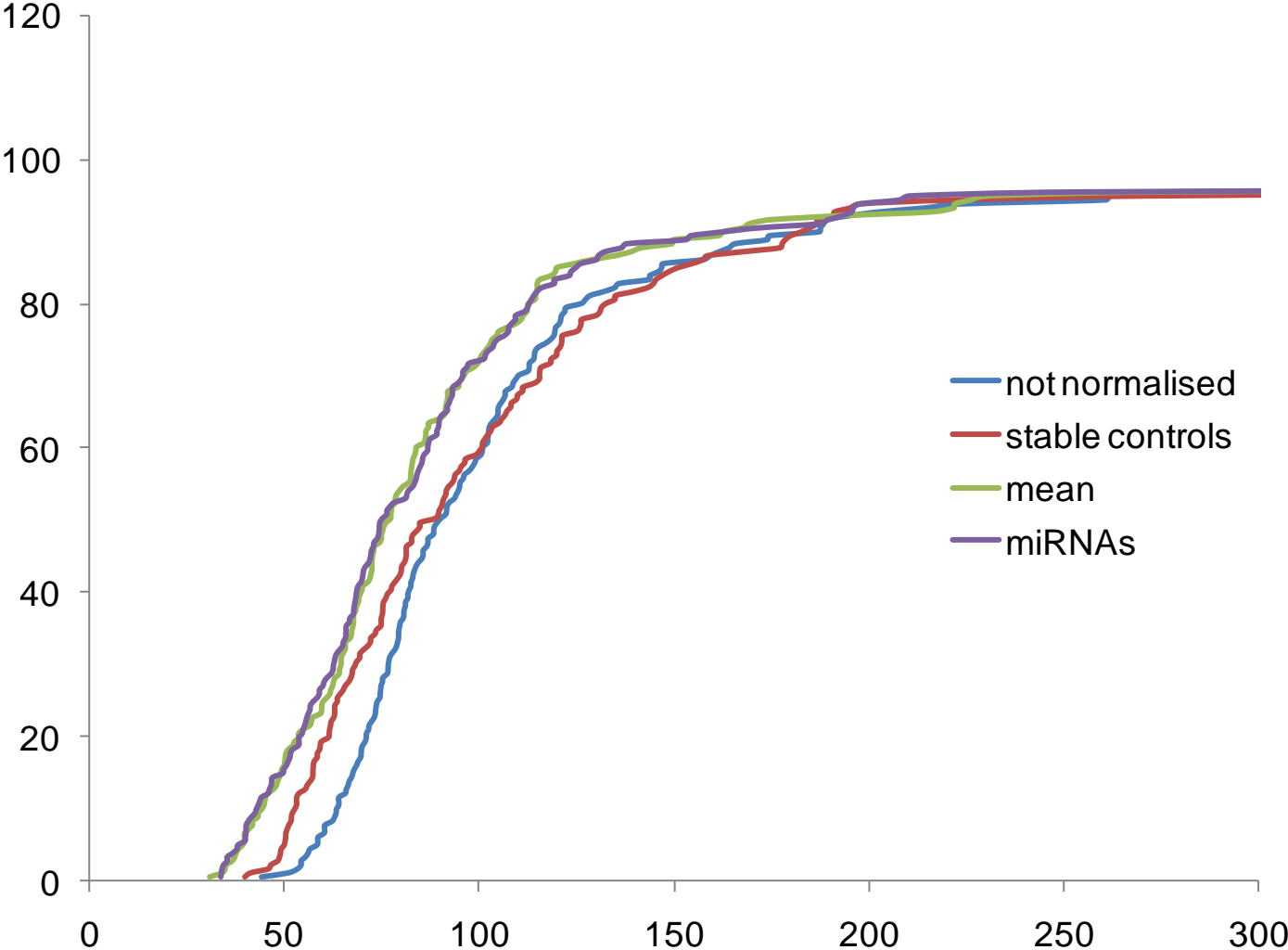




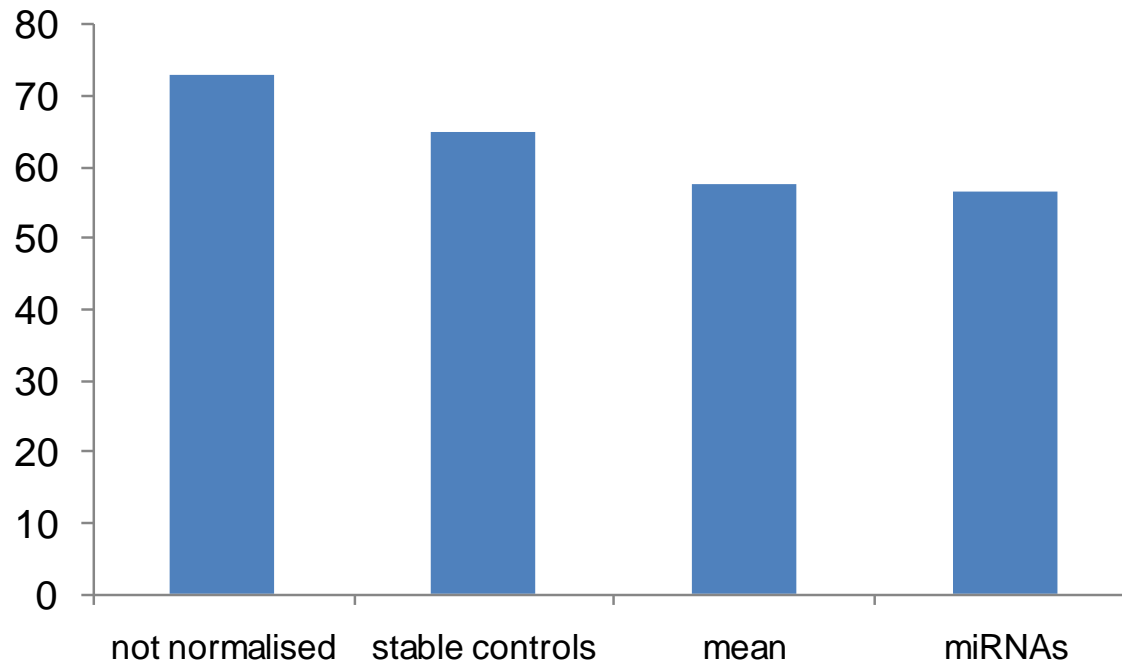
# normal tissues



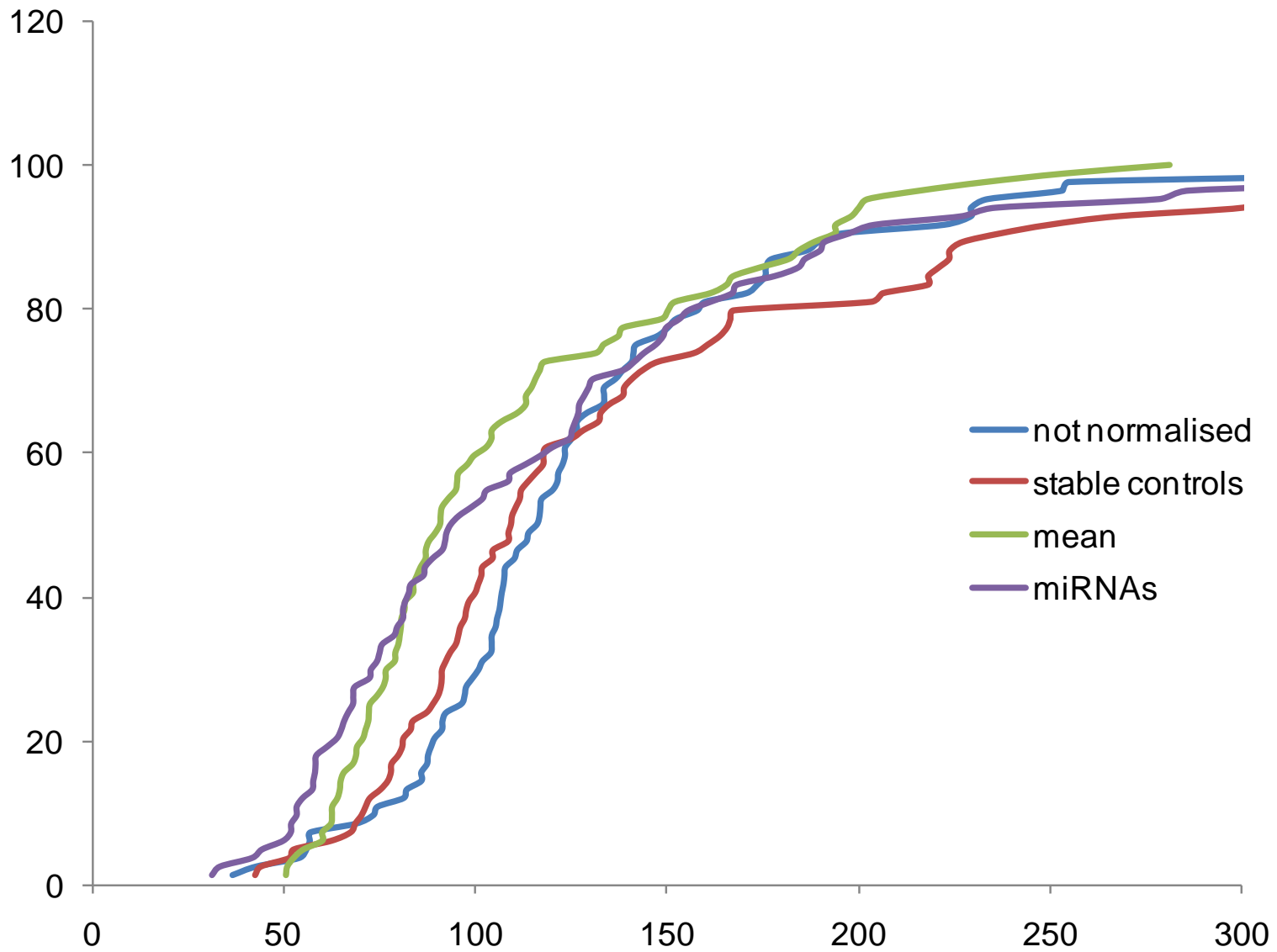
# neuroblastoma

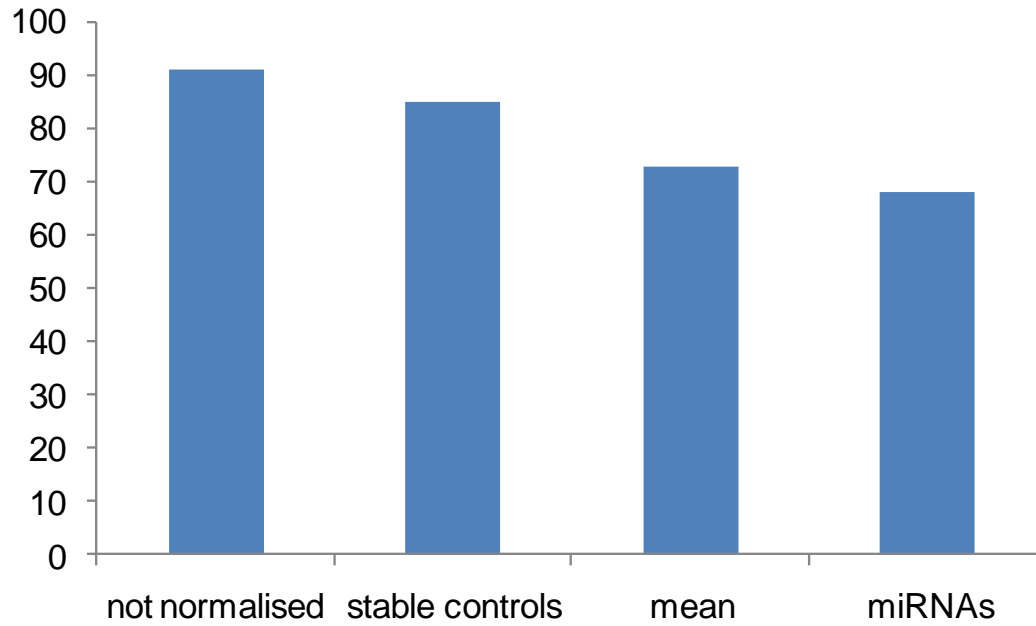


# neuroblastoma

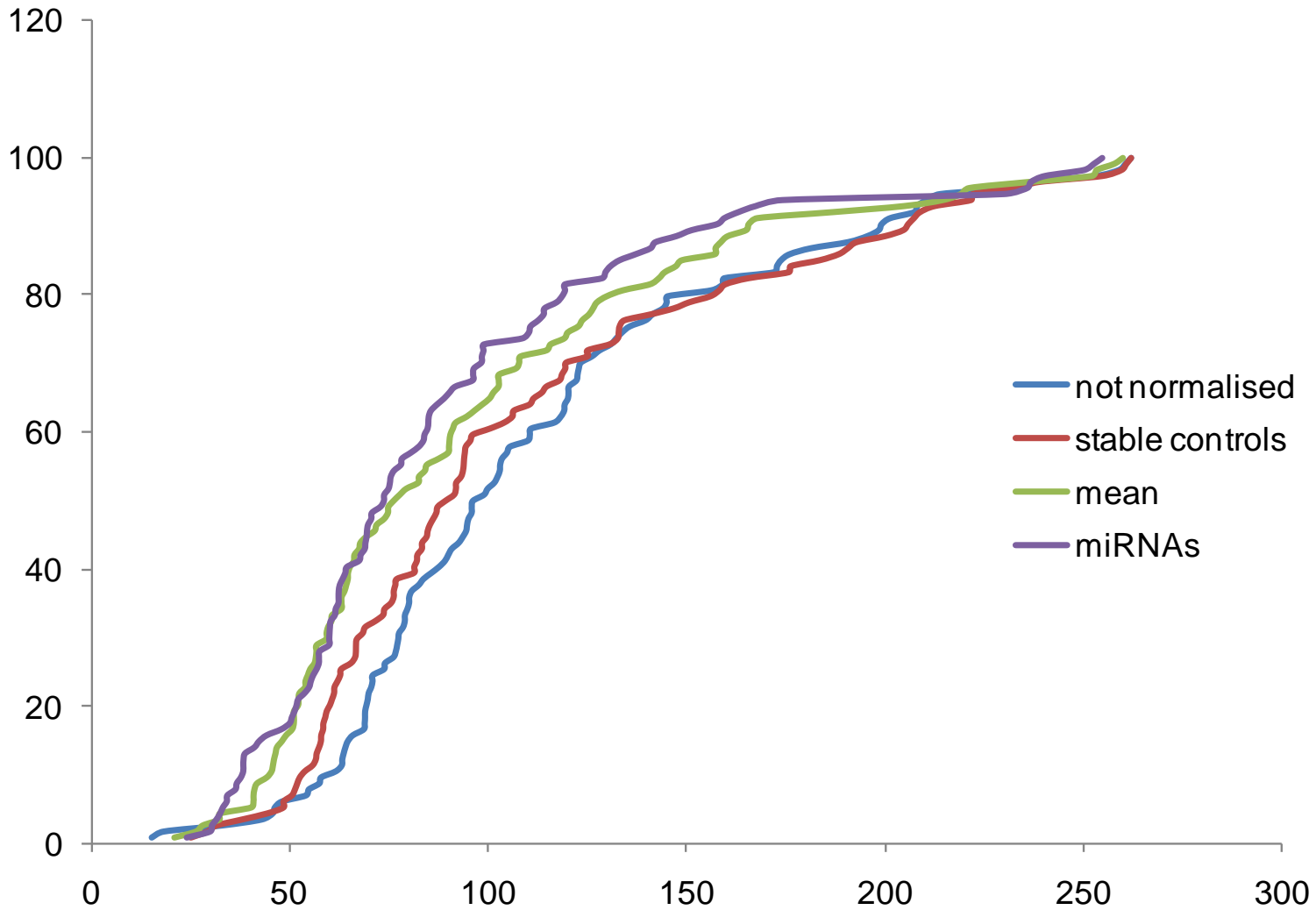


# T-ALL

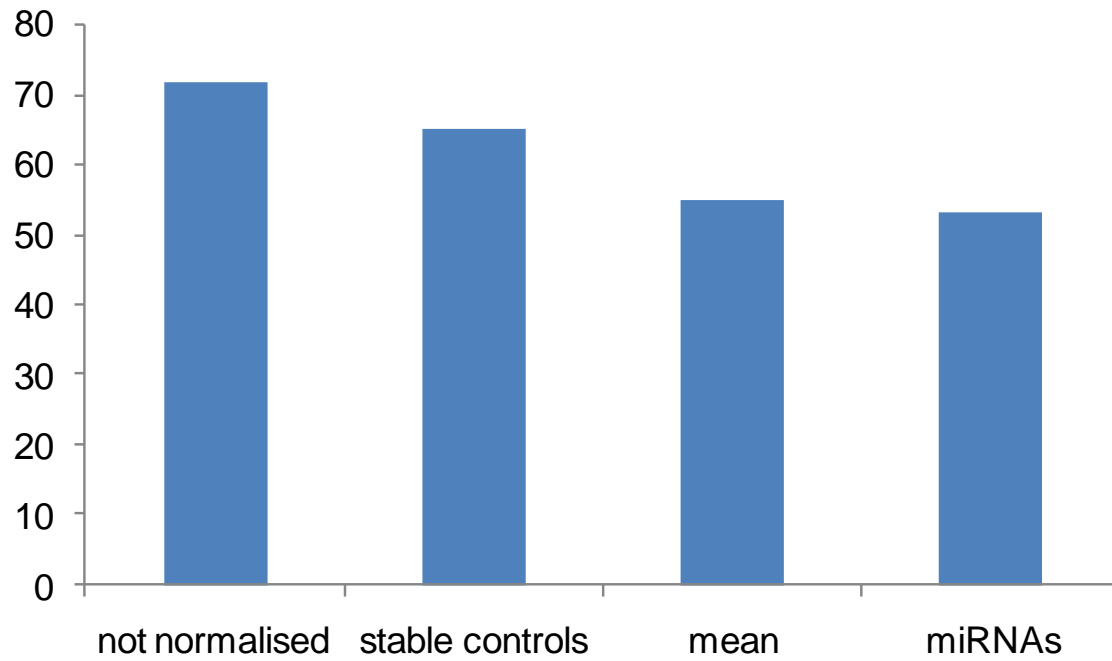




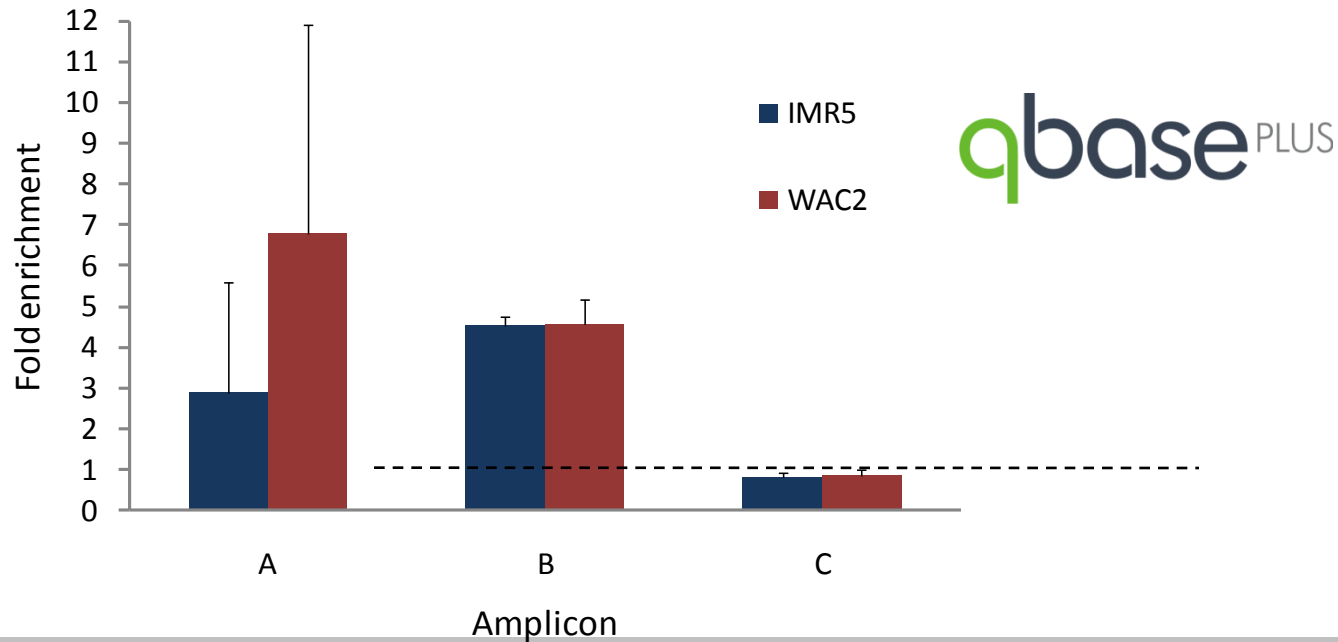
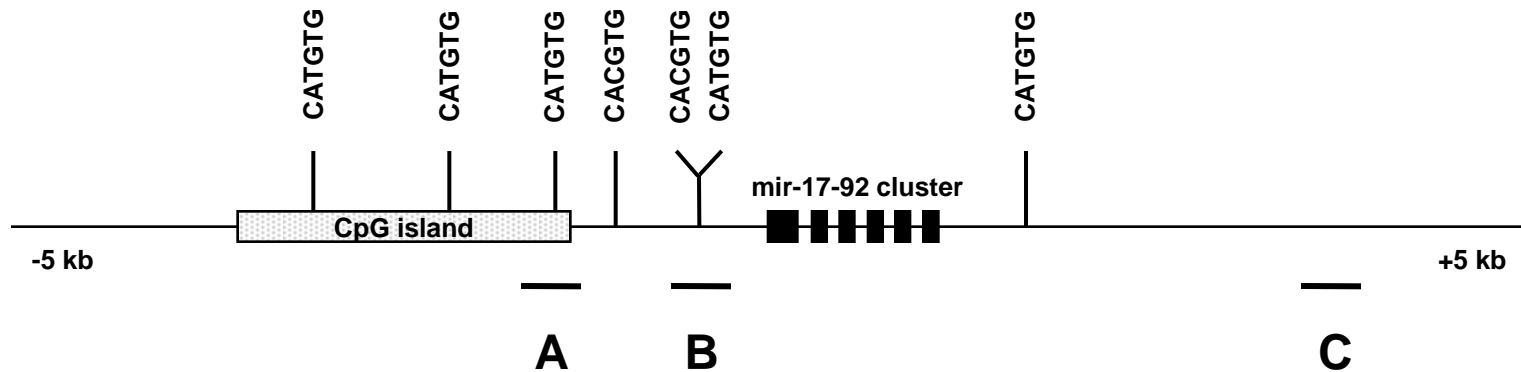
# normal tissues



# normal tissues

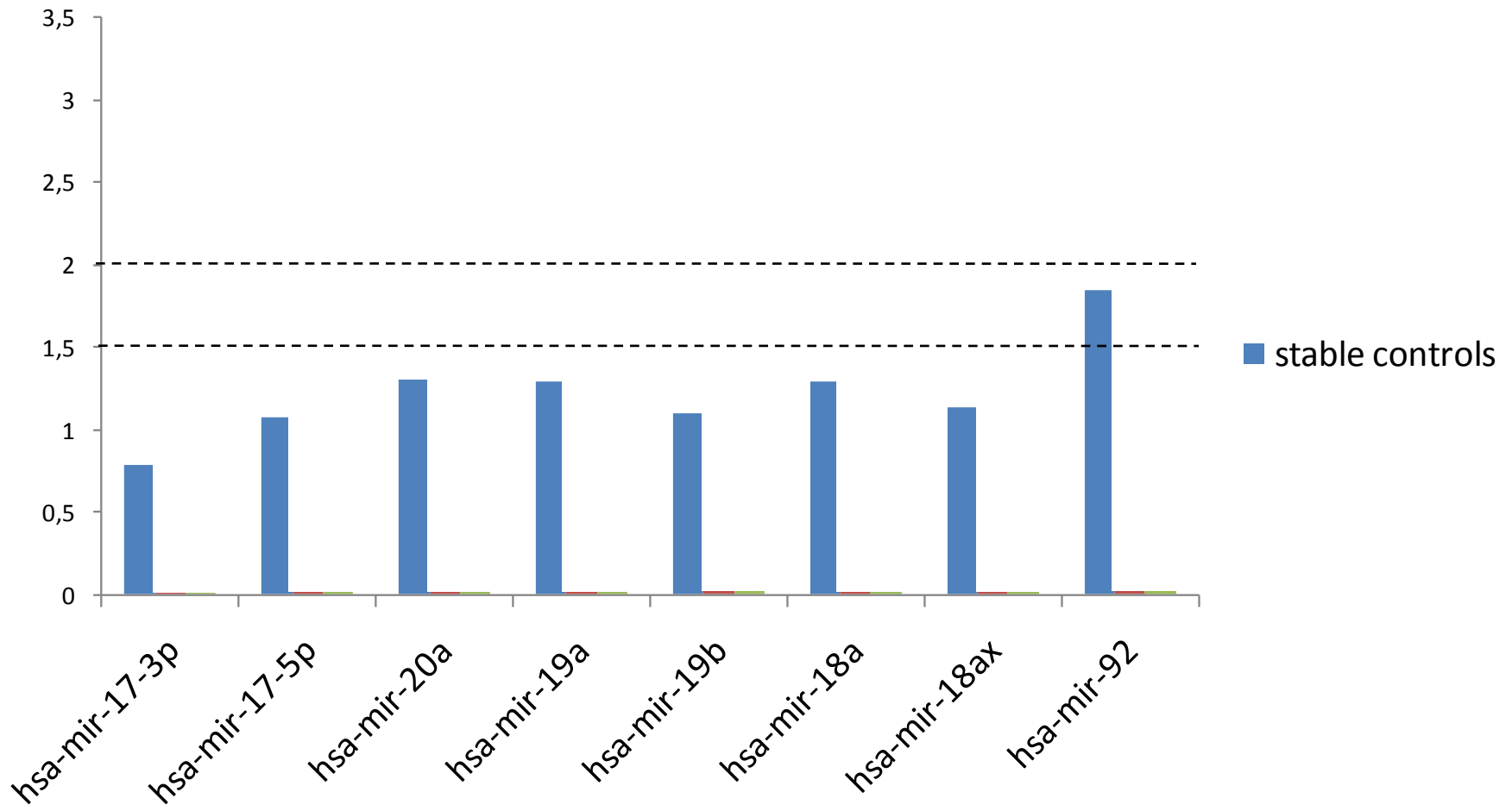


- MYCN binds to the mir-17-92 promoter



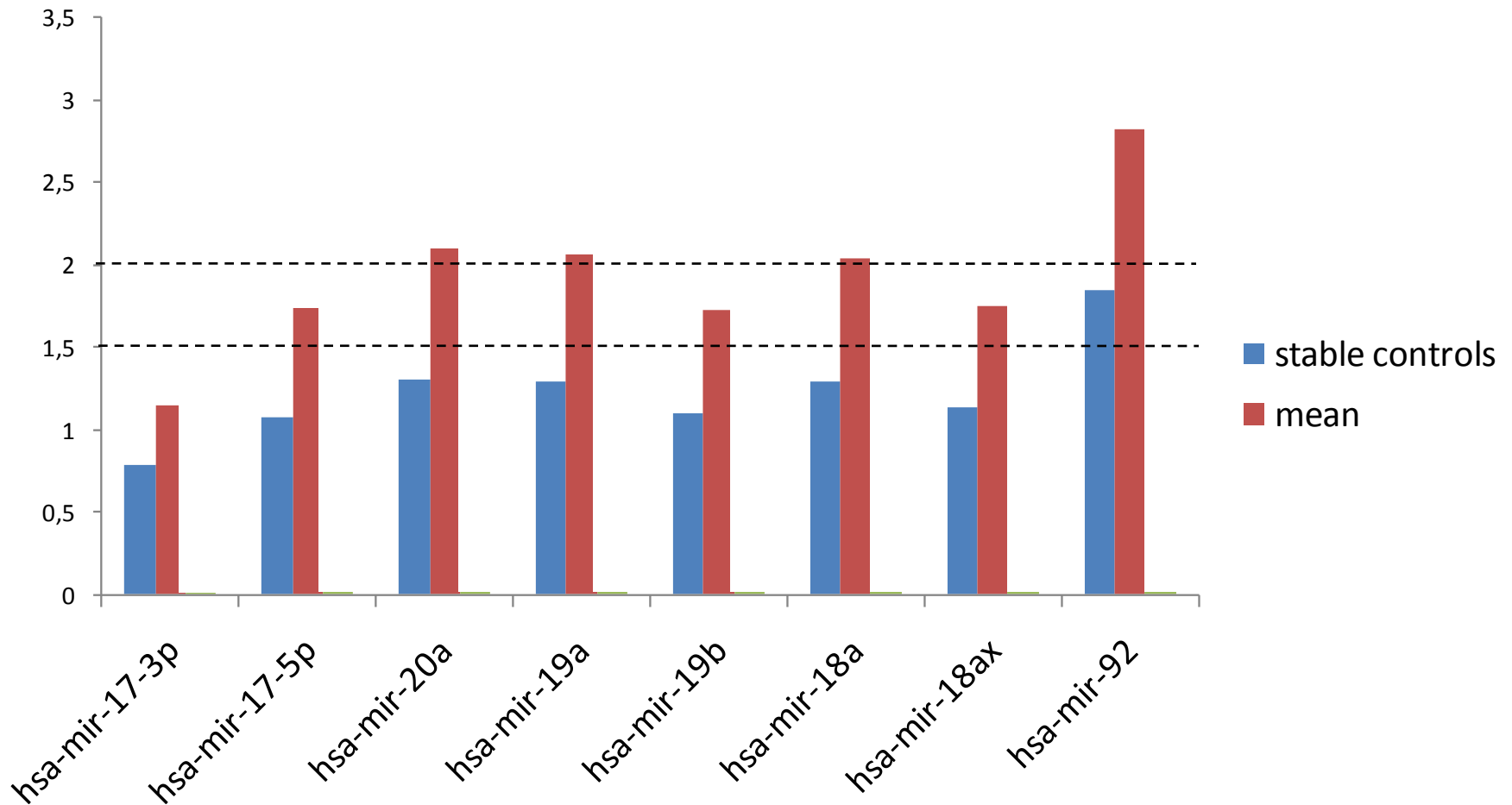
# biological validation

■ choice of normalization strategy influences differential miRNA expression



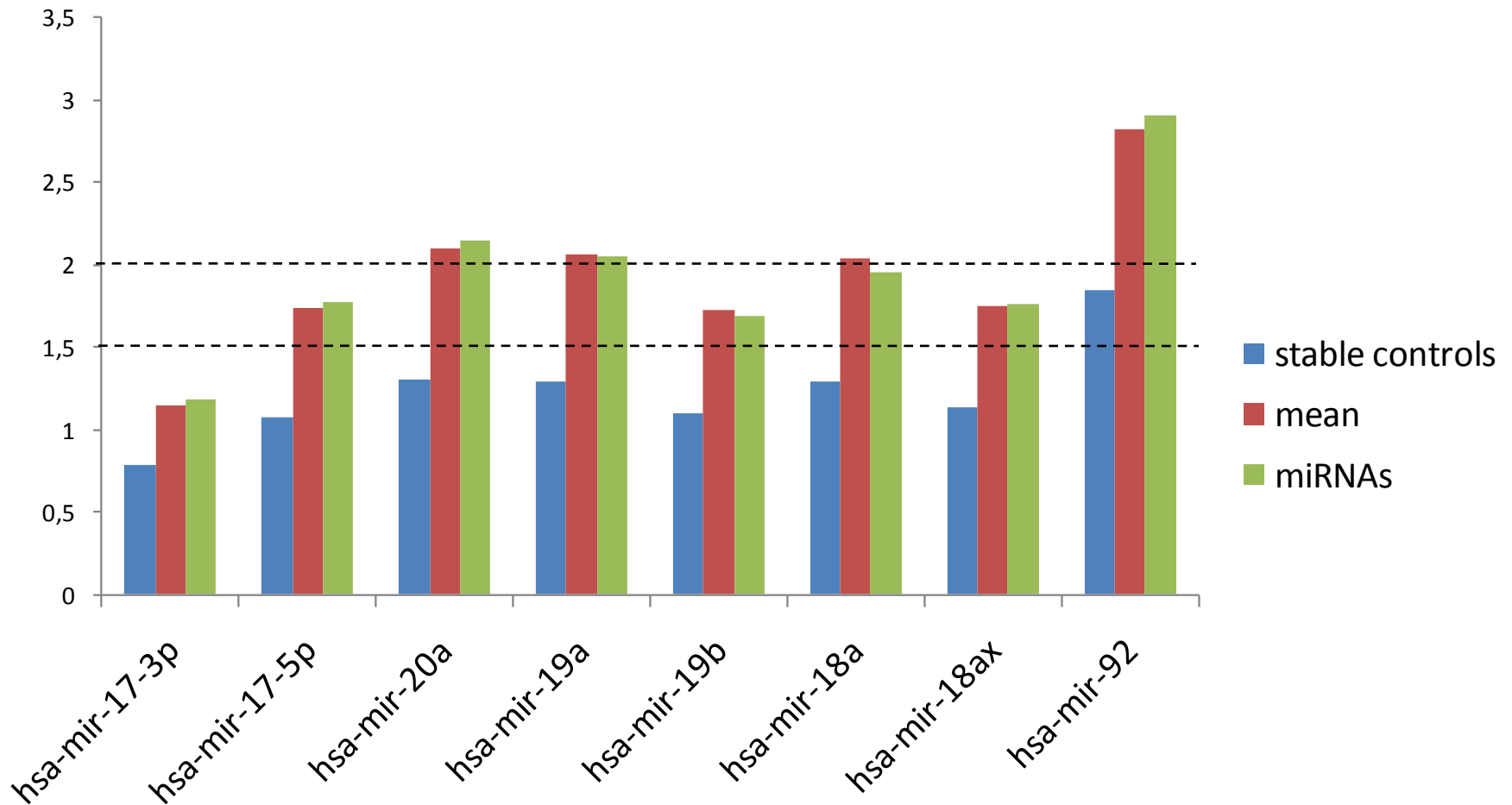
# biological validation

■ choice of normalization strategy influences differential miRNA expression

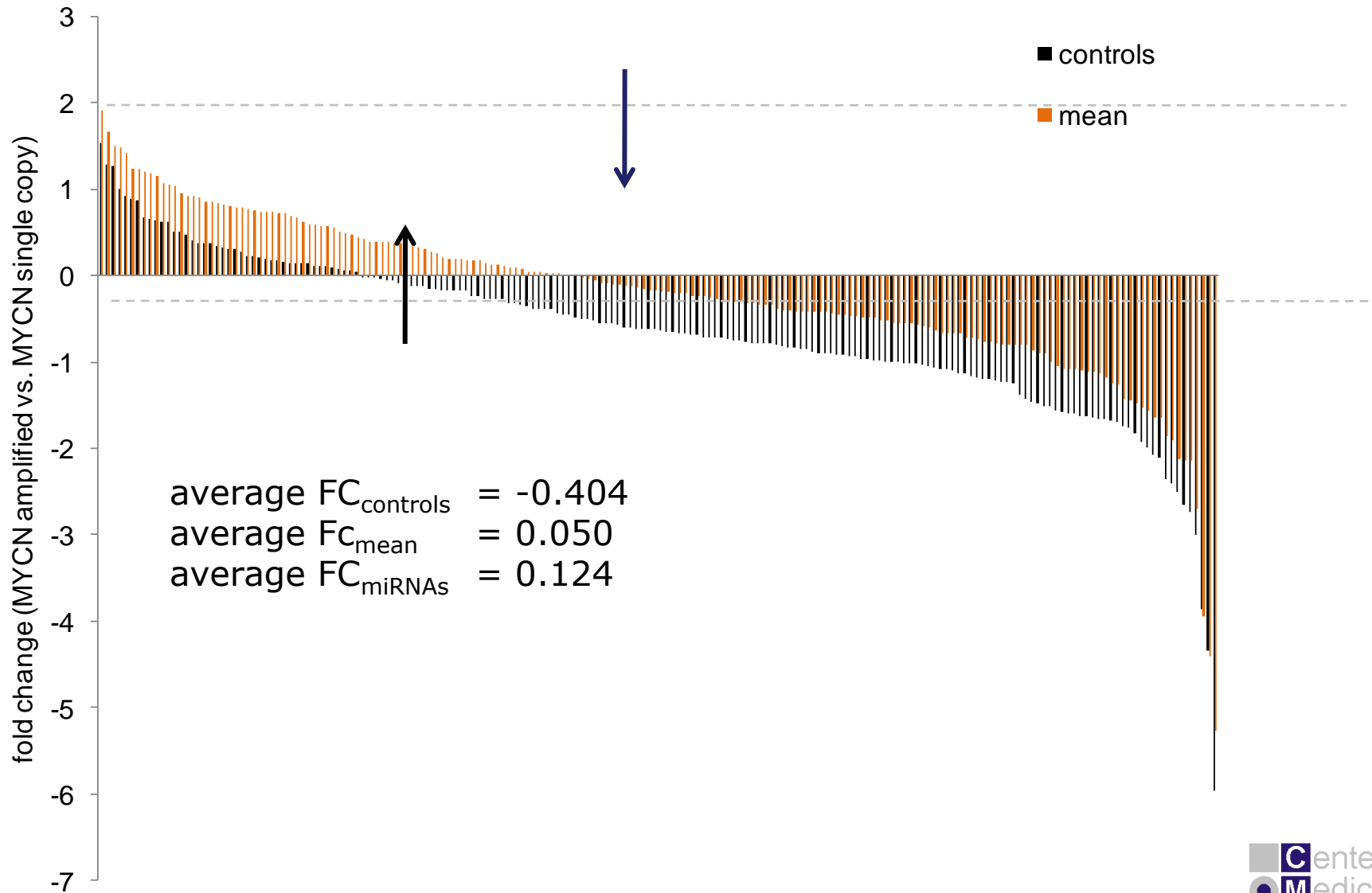


# biological validation

■ choice of normalization strategy influences differential miRNA expression



# balanced differential expression



- 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
    - o *mean*
    - o *multiple stable endogenous controls*

# acknowledgments

- miRNA, T-UCR
  - **Pieter Mestdagh**
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  - Justin Nuytens
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