



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

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Benelux qPCR symposium  
Ghent, Belgium

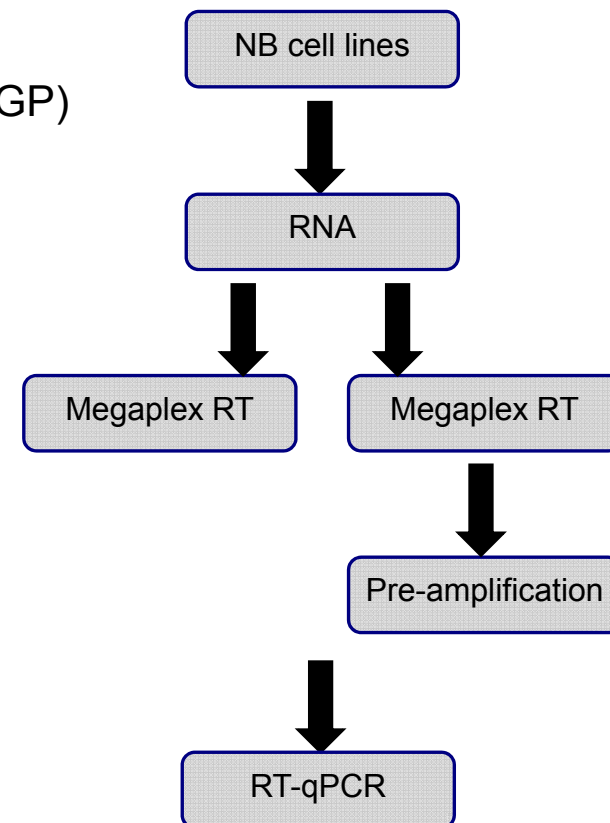


## overview

- platform evaluation
- applications in cancer research
- conclusions

## platform evaluation

- objectives
  - Evaluation of miRNA expression profiles using megaplex reverse transcription and optional pre-amplification
- experimental setup
  - 3 neuroblastoma cell lines (NBL-S, IMR-32, NGP)
  - Total RNA extraction (miRNeasy)
  - Megaplex reverse transcription (450 miRNAs)
  - Pre-amplification (14 cycles) of RT product
  - RT-qPCR expression profiling
- input RNA
  - Megaplex: 400ng total RNA
  - Megaplex + PreAmp:
    - 10ng total RNA
    - 1ng total RNA



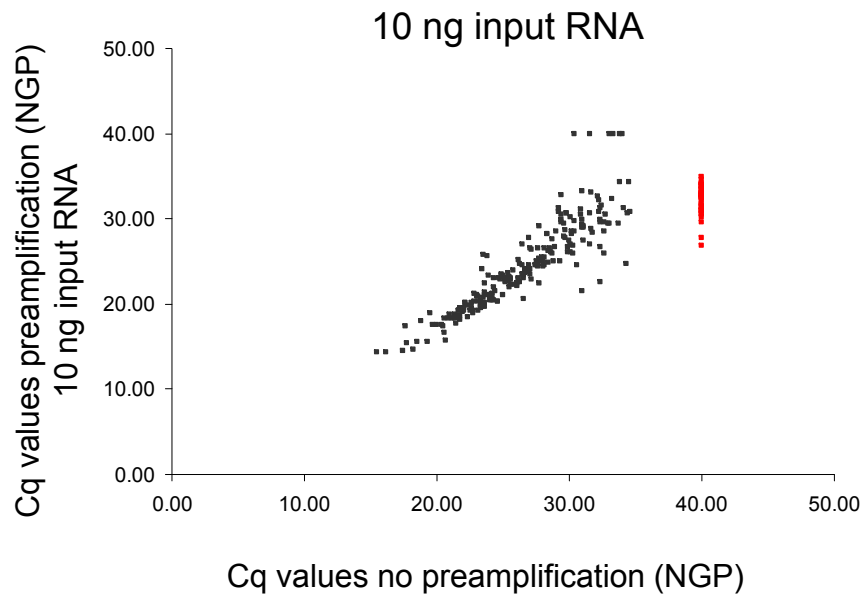
## platform evaluation

- RT-qPCR
  - qPCR plate setup: Gene Maximization
    - A different miRNA in each well of a 384 well plate (no replicates)
    - 1 sample per 384 well plate
  - qPCR reactions on 7900HT
  - Plate calibration
    - Calculation of average Cq for each 384 well plate
    - Equalization of averages
    - Undetermined values were assigned a Cq value of 40

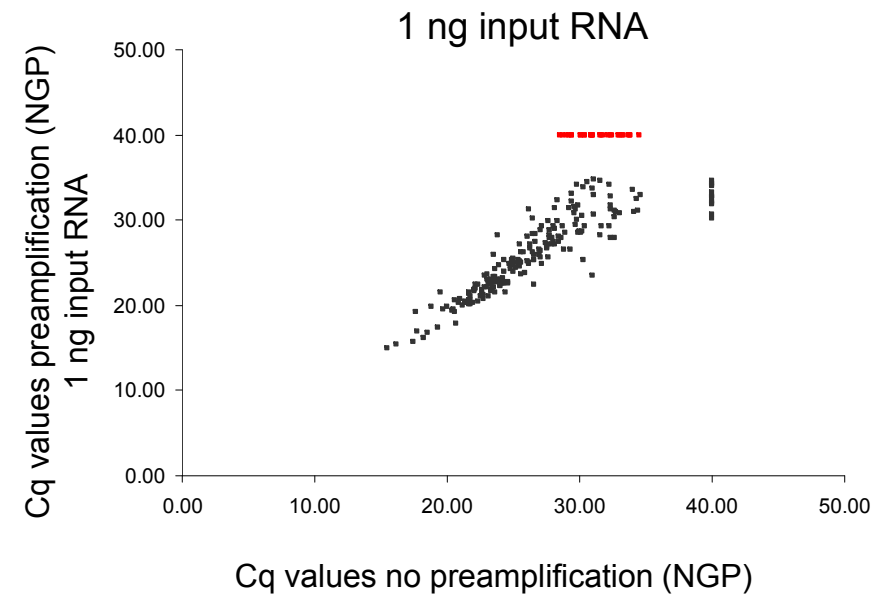


## platform evaluation

- minimal pre-amplification bias and sensitivity
  - Cq-Cq plots (pre-amplification vs. no pre-amplification)



*pre-amplification enables the detection of 'extra' miRNAs*



*reducing the amount of input RNA results in a loss of detectable miRNAs*

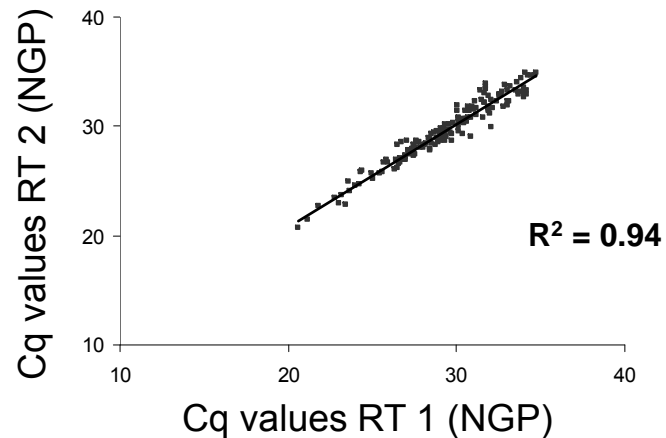
## platform evaluation

- amount of input RNA
  - Megaplex: 400 ng total RNA for 384 miRNAs → ~1 ng/miRNA
  - PreAmp: (assuming amplification efficiency of 100%)
    - 1 ng: ~10 ng/miRNA
    - 10 ng: ~100 ng/miRNA
- reverse transcription efficiency
  - 1ng total RNA ≈ 30 cells
  - For low abundant miRNAs (1 copy or less per cell), Megaplex RT input ≈ 30 copies (or less) → inefficient reverse transcription

## platform evaluation

- sources of variation

- RT reaction



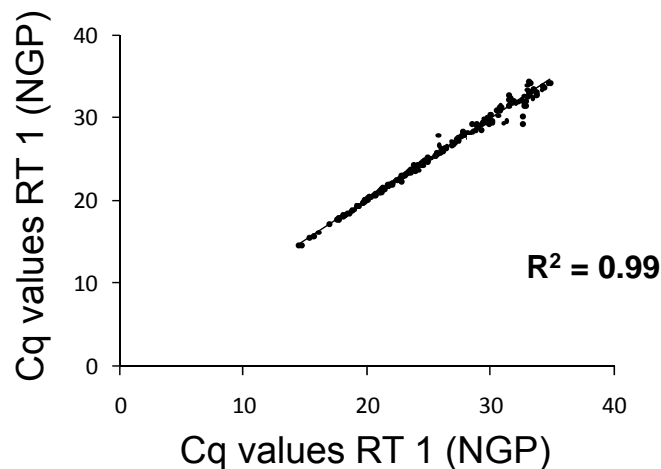
### RT variability for low copy RNA molecules

highly expressed miRNAs ( $Cq < 25$ ):  $R^2 = 0.872$

moderately expressed miRNAs ( $25 < Cq < 30$ ):  $R^2 = 0.806$

low abundant miRNAs ( $Cq > 30$ ):  $R^2 = 0.685$

- liquid handling and instrumentation



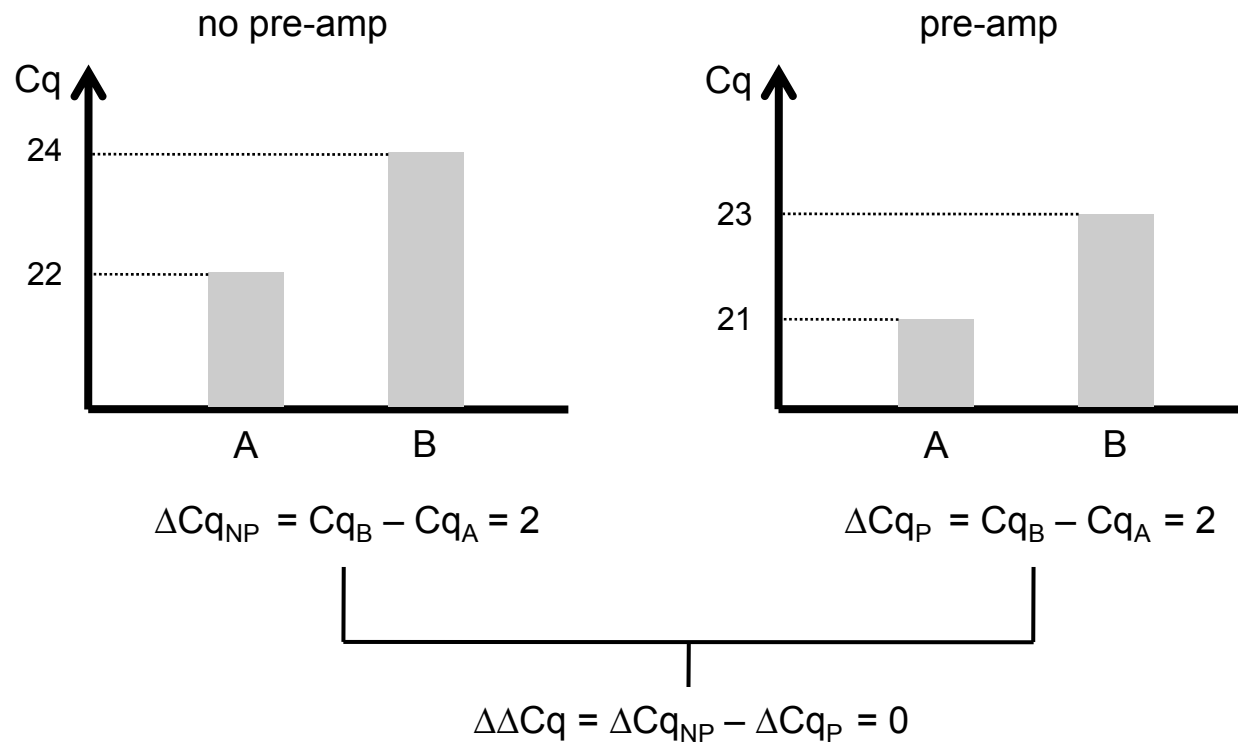
minor effect on observed variation

## platform evaluation

- preservation of differential miRNA expression

*Is differential miRNA expression maintained after introducing pre-amplification?*

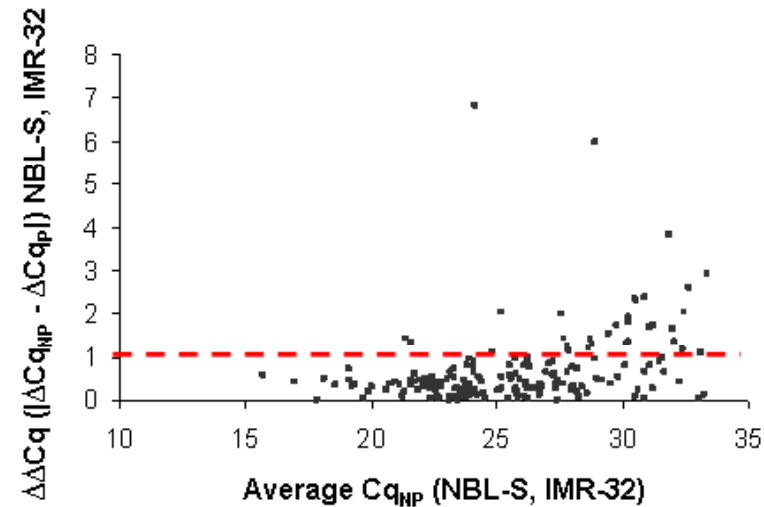
$\Delta\Delta Cq$  method:



## platform evaluation

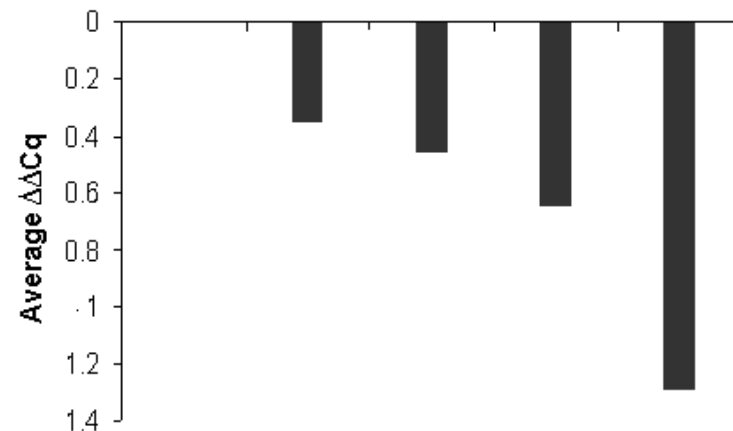
- preservation of differential miRNA expression

*Is differential miRNA expression maintained after introducing pre-amplification?*



$\Delta\Delta Cq < 1$ : 80%

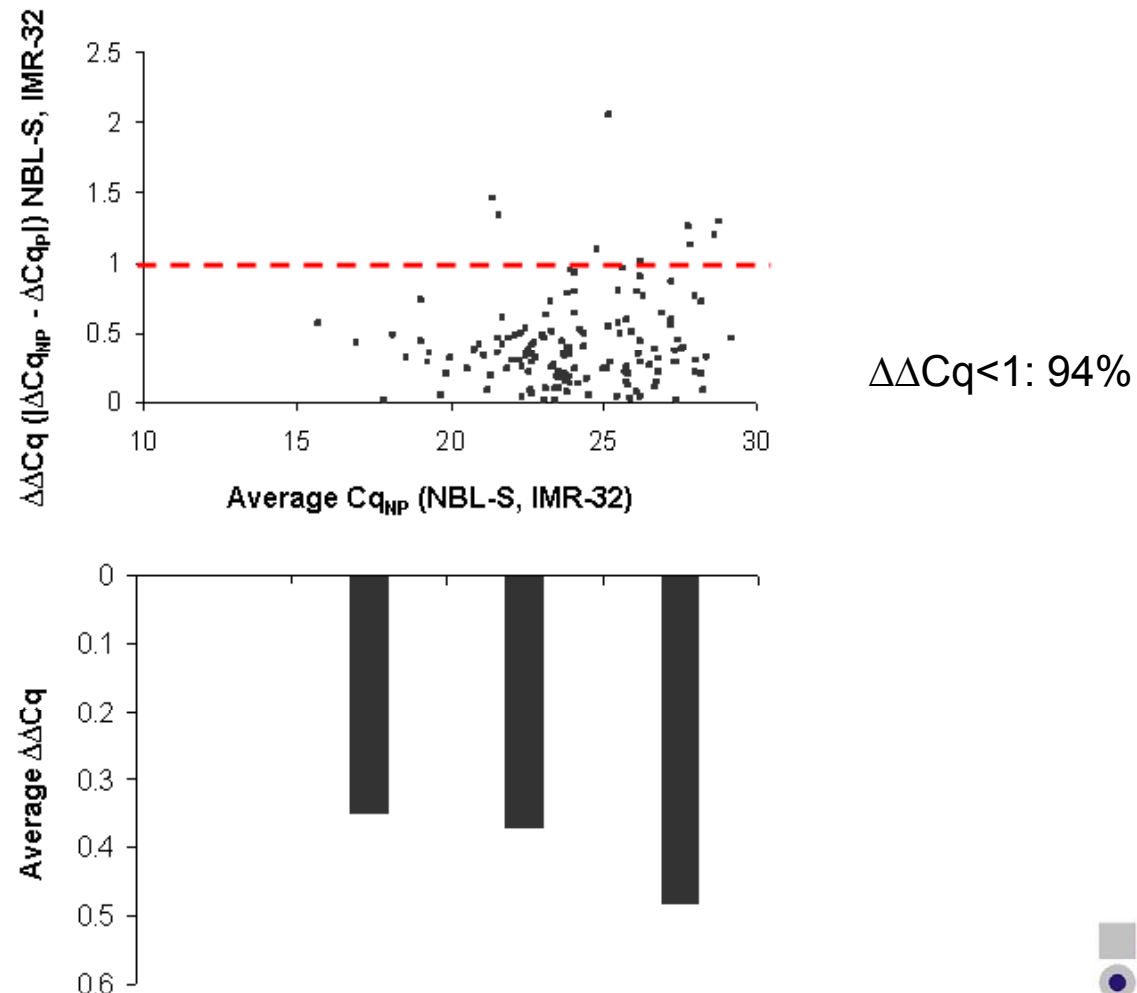
$\Delta\Delta Cq < 0.5$ : 75%



## platform evaluation

- preservation of differential miRNA expression

*Is differential miRNA expression maintained after introducing pre-amplification?*



$\Delta\Delta Cq < 1$ : 94%

## platform evaluation

- preservation of differential miRNA expression

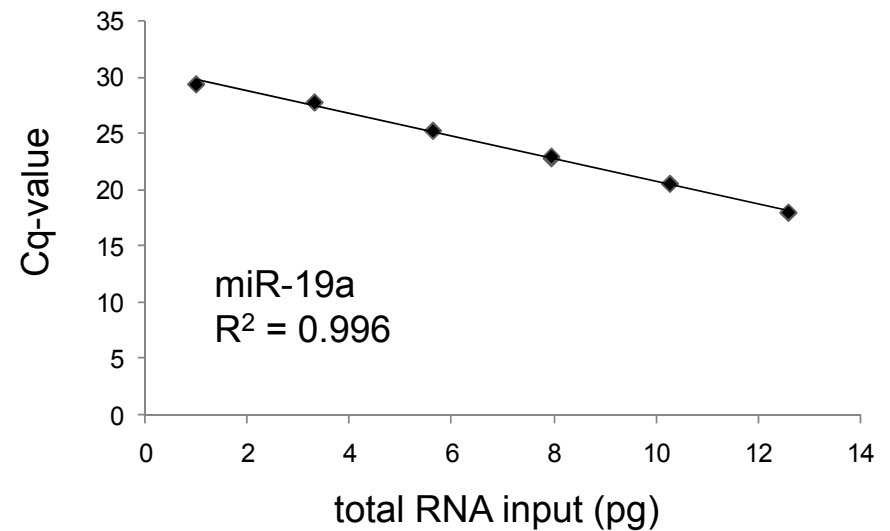
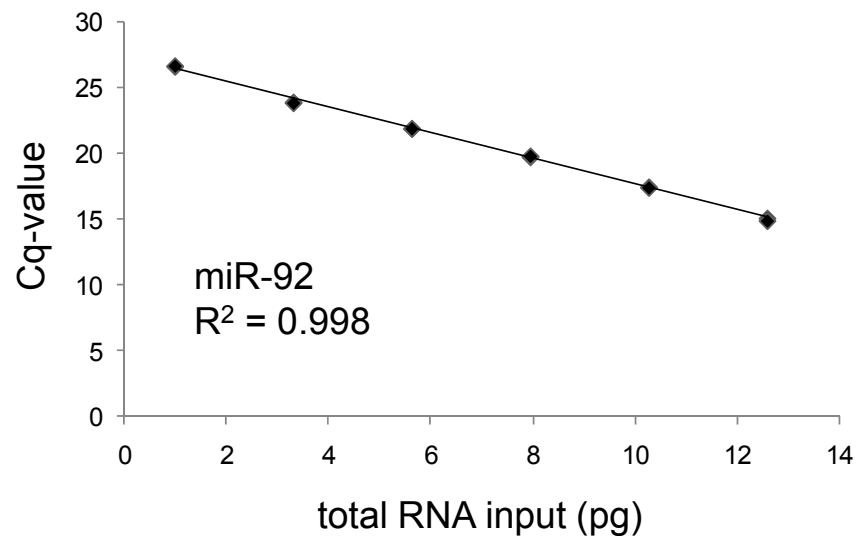
*Is differential miRNA expression maintained after introducing pre-amplification?*

- expression level dependent variation – partly attributable to RT variation
- majority of miRNAs with  $\Delta\Delta Cq > 1$  are differentially expressed irrespective of quantification method

example: mir-299-5p:  $\Delta Cq_{NP} = -8.3$   
 $\Delta Cq_P = -6.5$

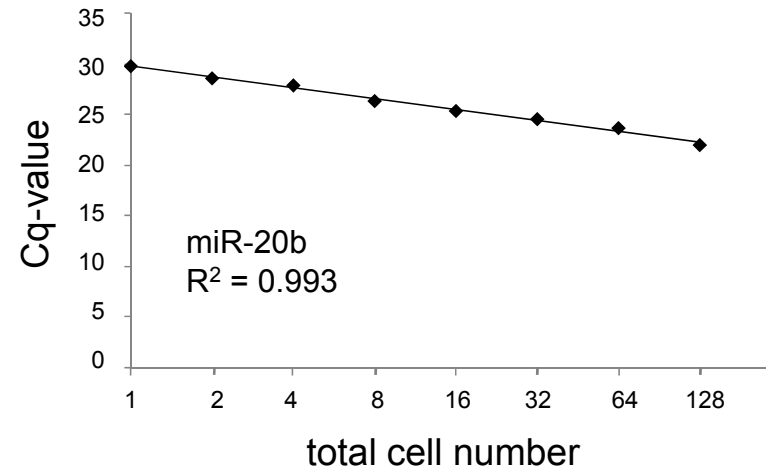
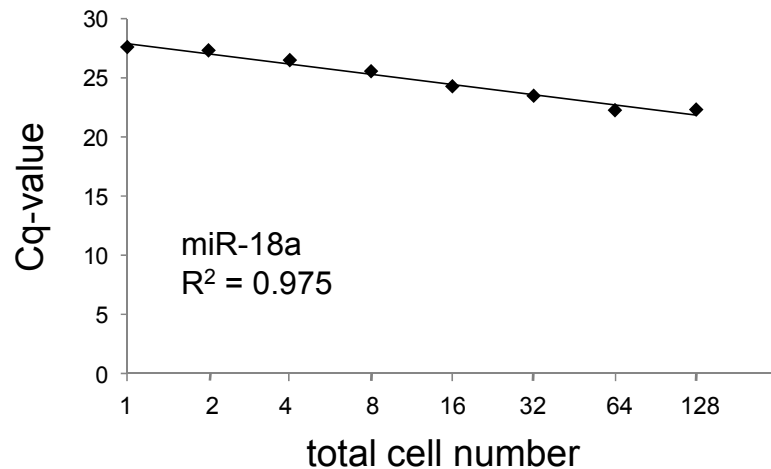
## platform evaluation

- single cell profiling
  - detection linearity for total RNA dilution series (2 pg – 6250 pg)



## platform evaluation

- single cell profiling
  - expression profiles should be readily obtainable from total cell lysate
  - microdissection of neuroblastoma cancer cells
  - detection linearity for limited cell dilution series (1-2-4-8-16-32-64-128)



## overview

- platform evaluation
  - megaplex RT – a one step miRNA cDNA synthesis
  - pre-amplification – reducing the amount of input RNA
- applications in cancer research
- conclusions

## applications

- applications of the miRNA profiling platform

TF regulated  
miRNAs

- EVI1
- MYCN

epigenetically  
regulated miRNAs

- methylation
- acetylation

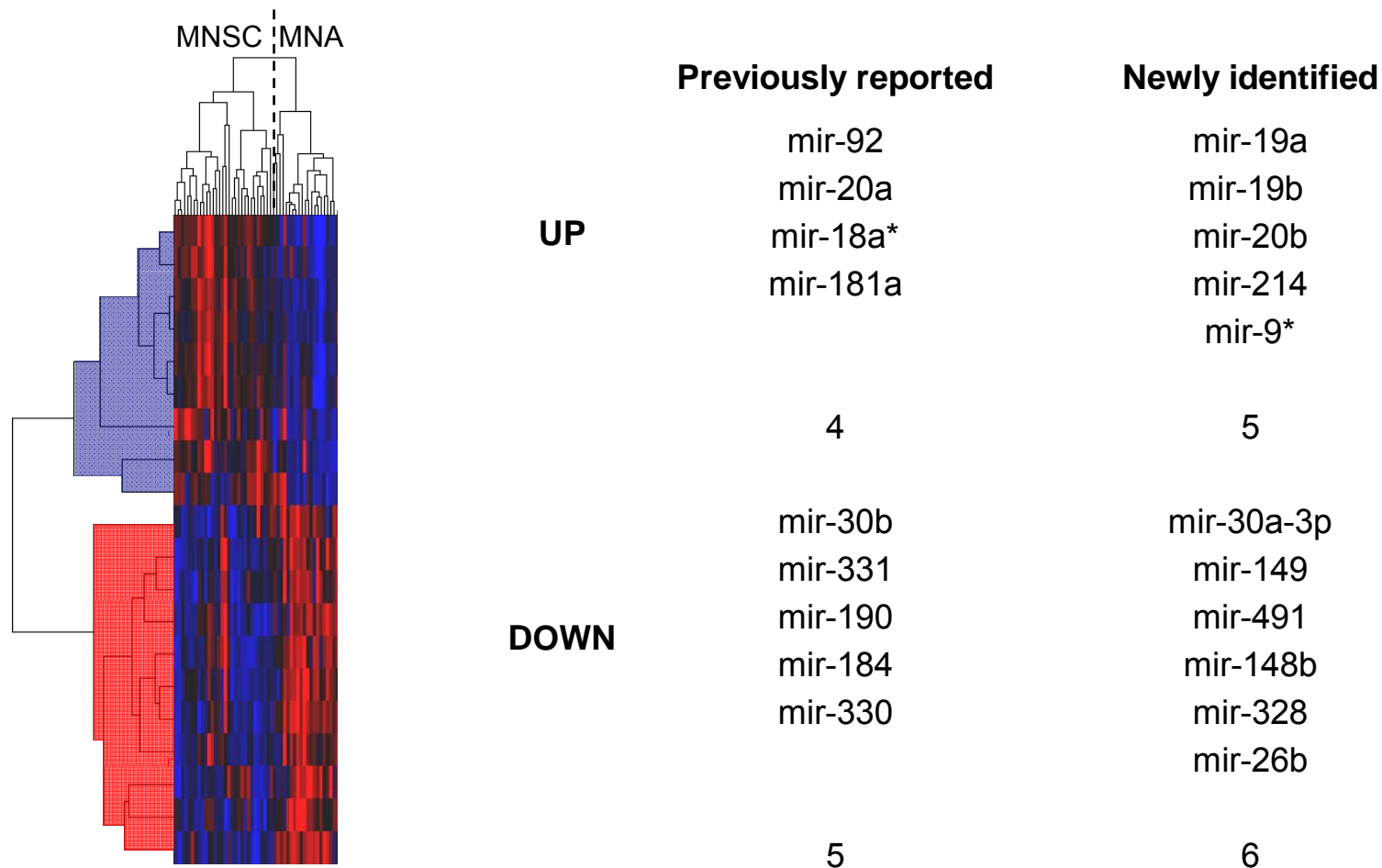
miRNA  
profiling  
platform

cancer stem cell  
specific miRNAs

prognostic miRNA  
signatures

## applications

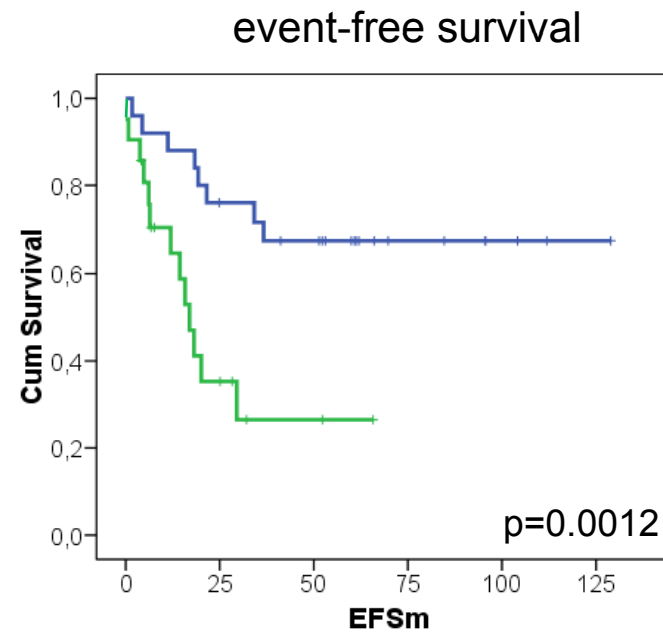
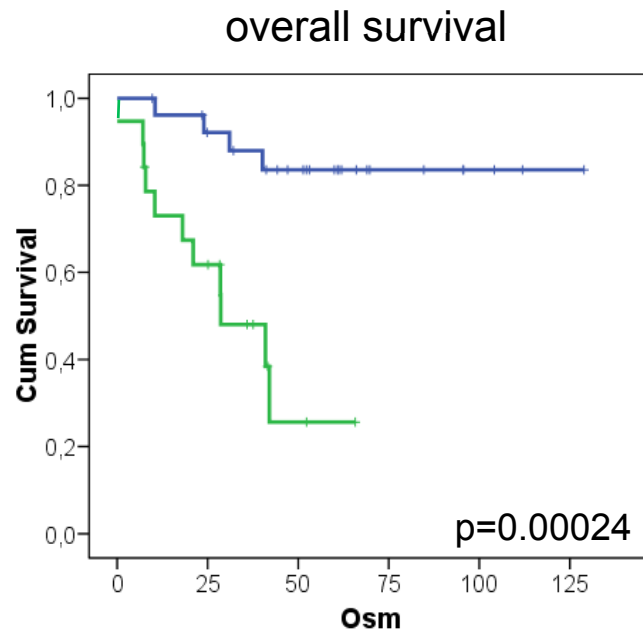
- identification of MYCN regulated miRNAs
  - miRNA profiling of 23 MYCN amplified and 33 MYCN single copy tumours
  - differential expression analysis (Mann-Whitney,  $p < 0.05$ )



## applications

- prognostic power of MYCN regulated miRNAs in neuroblastoma

*single miRNA signature as a powerfull predictor of OS and EFS*



## conclusions

- Megaplexing of stem-loop RT primers significantly increases miRNA profiling throughput in an RT-qPCR setting
- Introduction of a pre-amplification step increases detection sensitivity and allows for accurate miRNA profiling on the single cell level
- The introduction of a highly sensitive miRNA expression profiling platform lead to the identification of novel MYCN responsive miRNAs with prognostic power in neuroblastoma

## acknowledgements



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