The miRNA Revolution
Fall miRNA webinar series

miRNA quantification:
From experimental design through data analysis

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The second in a series of QIAGEN webinars on miRNA 2011

Welcome to the three-part webinar series on The miRNA Revolution

Webinar 1 :	**Meeting the challenges of miRNA research - An introduction to miRNA function & analysis**
Date:	**October 5th, 2011**
Speaker:	**Jonathan Shaffer, Ph.D.**

Webinar 2 :	**miRNA Quantification From experimental design through data analysis**
Date:	**October 12th, 2011**
Speaker:	**Subu Yerramilli, Ph.D.**

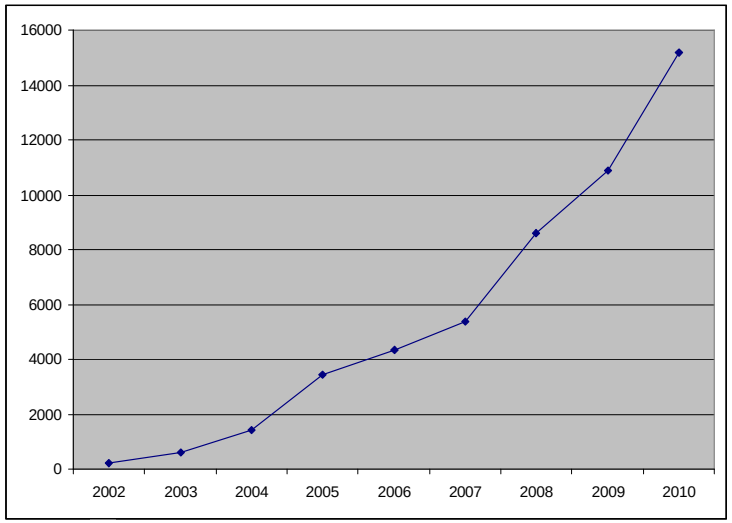
Webinar 3 :	**Profiling miRNA expression in Cells, FFPE, and Serum: on the road to biomarker development**
Date:	**October 19th, 2011**
Speaker:	**Eric Lader, Ph.D.**

miRNA quantification:
From experimental design through data analysis

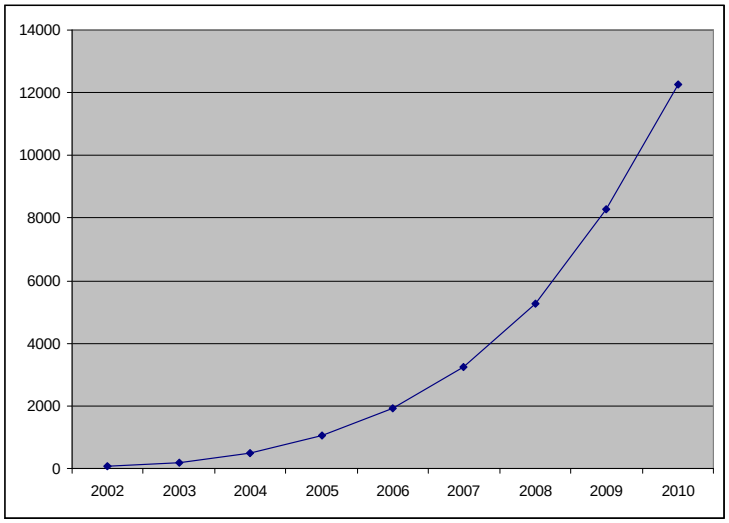
Topics to be covered

Challenges in miRNA quantification
The miScript System
Profiling the miRNome
Data Analysis

Why Quantify miRNA?



Known miRNAs in Sanger DB



miRNA publications in Medline

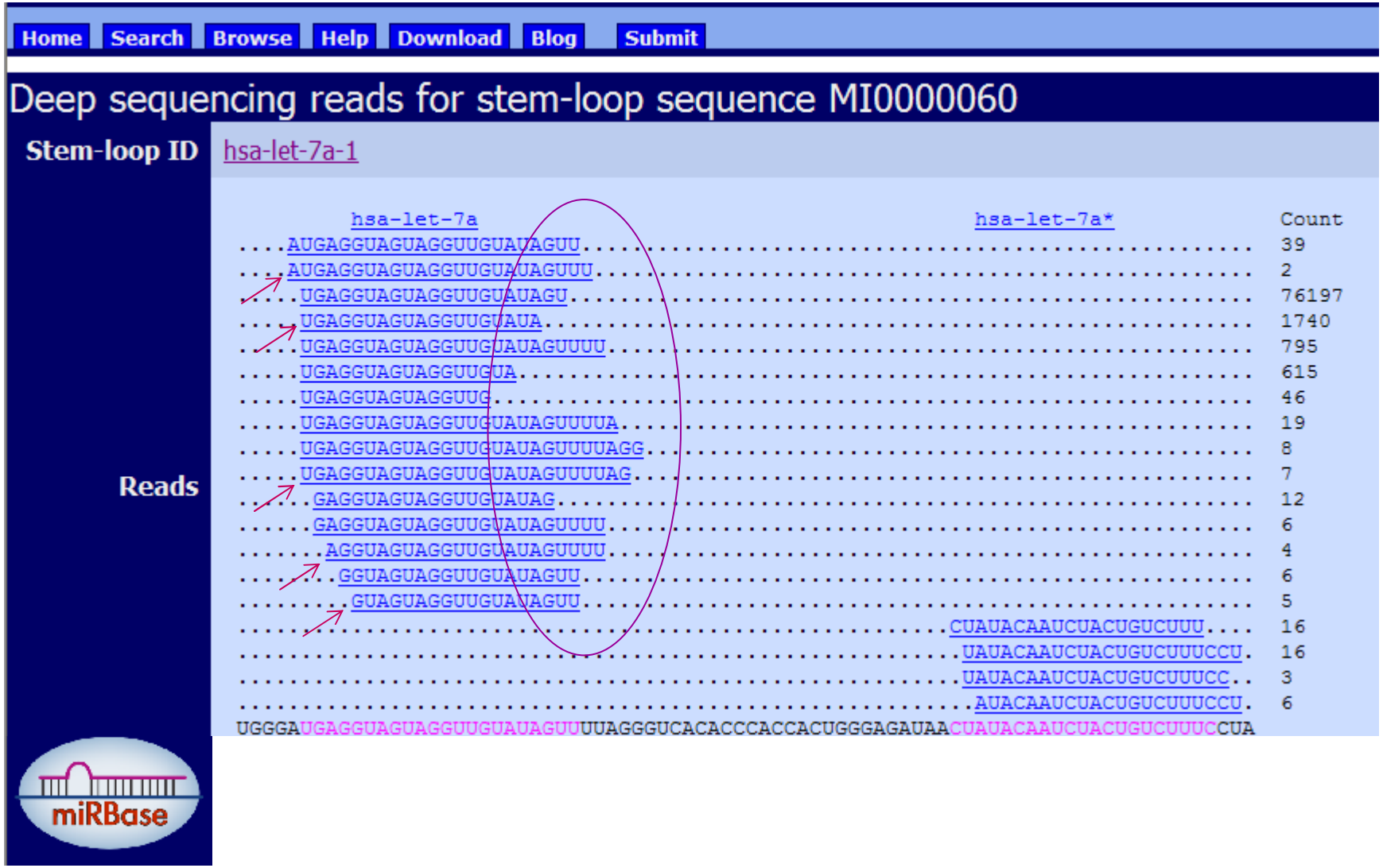
Virtually every publication included quantification experiments

Changes in miRNA correlated with gene expression changes in development, differentiation, signal transduction, infection, aging, and disease

miRNA Quantification Challenges

- Short sequences: ~16-26 nt
- Presence of miRNA isoforms
  - Near identical with ~1-3 nt differences
  - Can be any where along the sequence
- miRNAs with less than ideal GC% (very low or very high)
- Presence of repetitive sequences
  - hsa-miR-296-5p: AGGGCCCCCCCUCAAUCCUGU
  - hsa-miR-638: AGGGAUCGCGGGCGGGUGGCGGCCU
  - hsa-miR-940: AAGGCAGGGCCCCCGCUCCCC
  - mmu-miR-703: AAAACCUUCAGAAGGAAAGAA
  - mmu-miR-466b-5p: UAUGUGUGUGUACAUGUACAUA
  - mmu-miR-574-5p: UGAGUGUGUGUGUGUGAGUGUGU
- 3’-and 5’-end variations (Next slide)

3’- End & 5’-End Variations in miRNA



miRNA Quantification Strategies

- Micro array based approaches
  - Low specificity, needs further validation
- Expression analysis by deep sequencing
  - Expensive, and low throughput
  - Not practical for large number of samples
- Real time PCR based approaches
  - Fast, flexible, scalable, accurate, sensitive

Real-Time PCR Based Approach
miRNA 3'-End Dependent Reverse Transcription

e.g. hsa-Let-7a

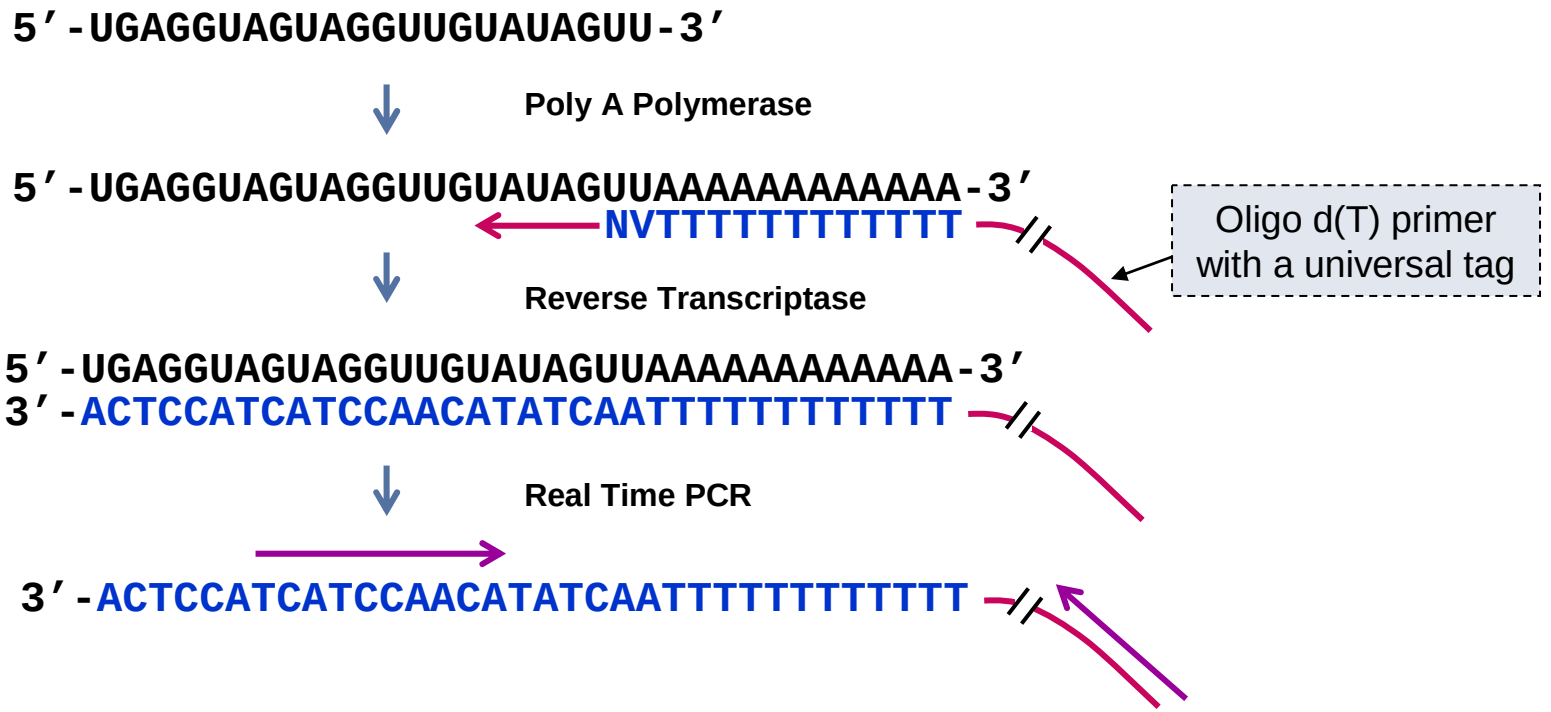
5'-UGAGGUAGUAGGUUGUAUAGUU-3'

TATCAA → **miRNA specific cDNA**

- Each miRNA detection requires a separate cDNA synthesis reaction
- A given RT primer could prime multiple miRNAs if these share the 3'-sequennces
- Doesn't address 3'-end Polymorphism

www.mirbase.org

Real-Time PCR-Based Approaches
Universal Reverse Transcription



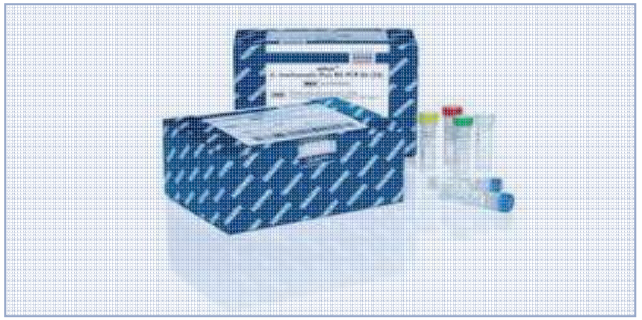
qPCR with
miRNA specific FWD primer
Universal Reverse Primer

- A single cDNA prepared per sample is sufficient to interrogate many miRNAs
- 3'-end polymorphism tolerated

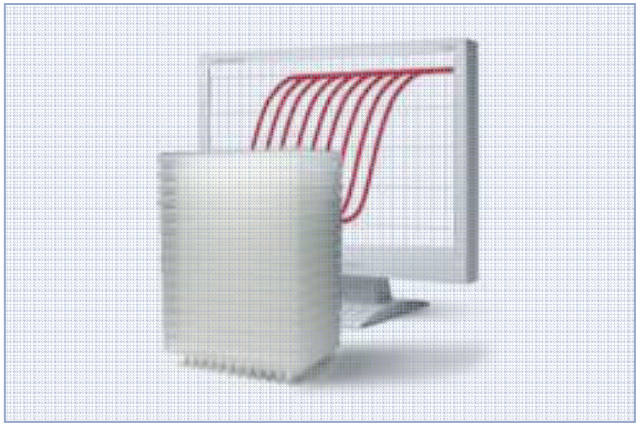
Next Generation miScript PCR System with miScript II RT

New

1. **miScript II RT kit**
   - ☐ miScript Reverse Transcriptase Mix
   - ☐ 5x HiSpec Buffer
   - ☐ 5x HiFlex Buffer
   - ☐ 10x Nucleics Mix
   - ☐ Water



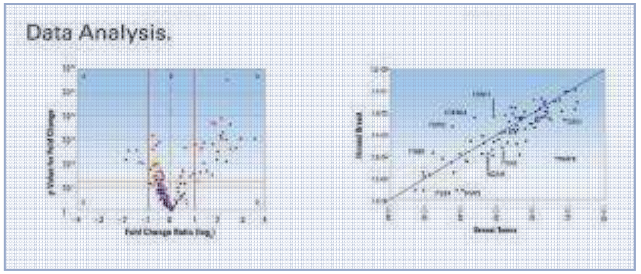
2. **miScript SYBR Green PCR Kit**
   - ☐ QuantiTect SYBR Green PCR MM
   - ☐ Universal Primer



3. **Assays**
   - ☐ miScript Primer Assays
   - ☐ miScript Precursor Assays
   - ☐ QuantiTect Primer Assays
   - ☐ miScript Controls (NEW)

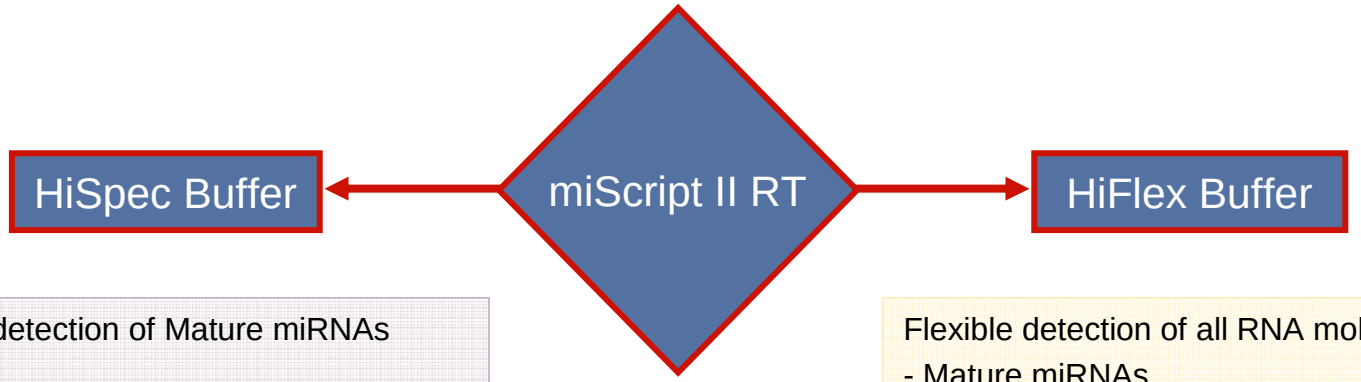
New

4. **miScript PCR Arrays**
   - ☐ Whole miRNome PCR Arrays
   - ☐ miScript miRNA PCR (Focused) Arrays



5. **Online Array Data analysis software**
   - ☐ **ACCESS AT NO COST**
   - ☐ **Data analysis made easy**

miScript II RT:
Dual Buffer System Offers Flexibility & Specificity



Specific detection of Mature miRNAs

Only mature miRNA detection is desired
Single Assays
miRNA PCR Arrays
miRNA profiling using miRNome Arrays

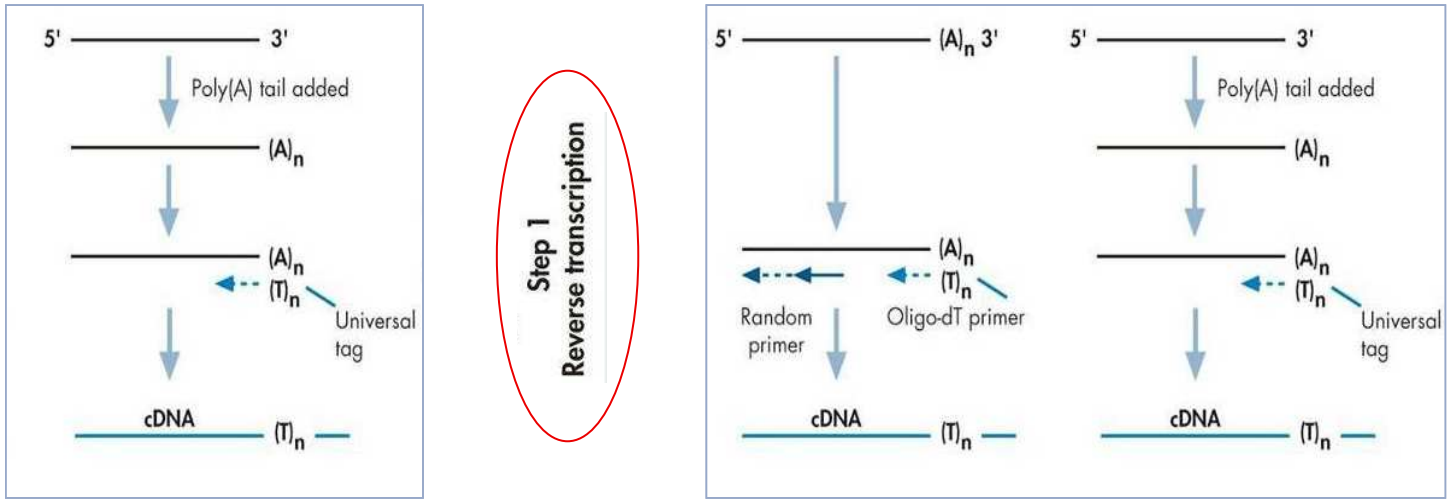
Flexible detection of all RNA molecules
- Mature miRNAs
- Precursor miRNAs
- mRNAs
- Other non coding RNAs

When mRNAs/precursors/other long non coding RNAs quantified in parallel with mature miRNAs

New miScript Controls work equally well in both buffer systems

Unique RT Buffer Formulation: Offers Flexibility, Sensitivity & Specificity

HiSpec Buffer ← miScript II RT → HiFlex Buffer

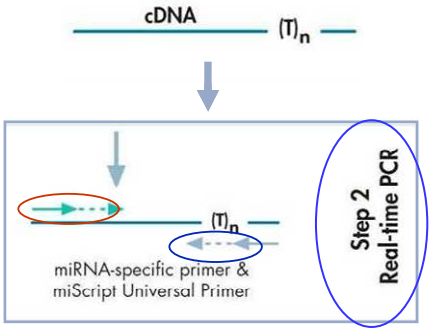


cDNA for the detection of:
small RNA molecules only

cDNA for the detection of:
mature miRNAs, Pre-miRNAs, mRNAs & Other nc RNAs

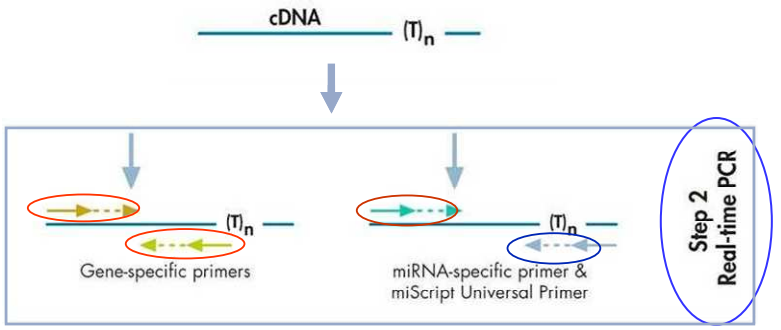
miScript PCR System:
Real time PCR Step

RT in HiSpec Buffer



miRNA detection
miScript Primer Assays
miScript miRNA PCR Arrays
miScript miRNome Arrays
miScript Controls

RT in HiFlex Buffer

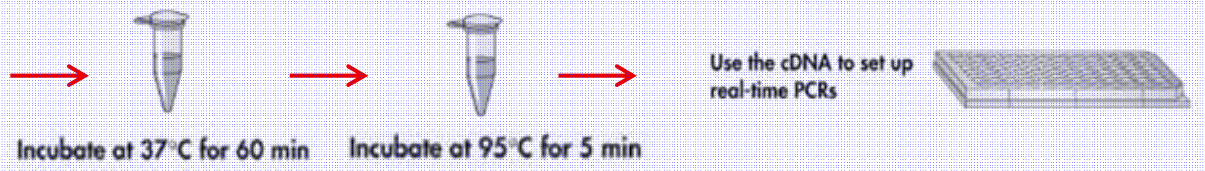


mRNA detection
QuantiTect Primer Assays

miRNA detection
miScript Primer Assays
miScript Controls

miScript PCR System workflow: Simple & Easy steps

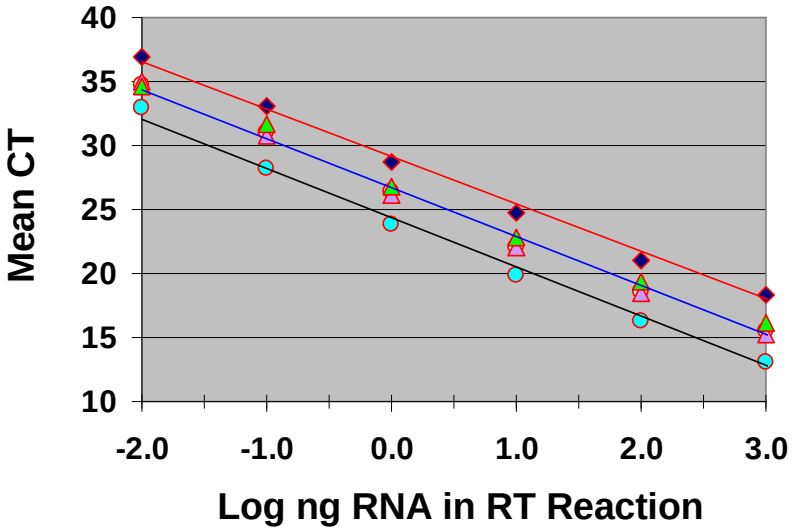
Mix RNA,
5x miScript HiSpec or HiFlex Buffer,
10x Nucleics Mix, RNase-free water &
miScript Reverse Transcriptase Mix



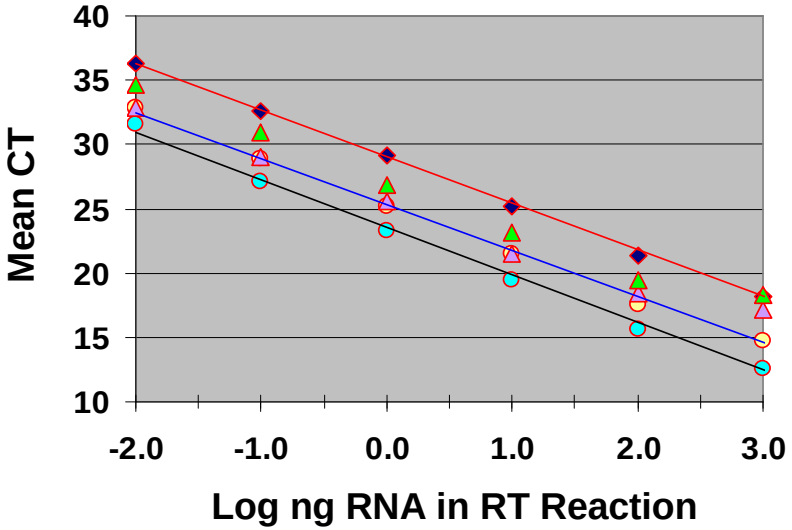
Linear cDNA Synthesis Over Five Logs Of Input RNA: With miScript II RT

Linear in the range of 10 pg to 1 µg

RT in HiSpec Buffer

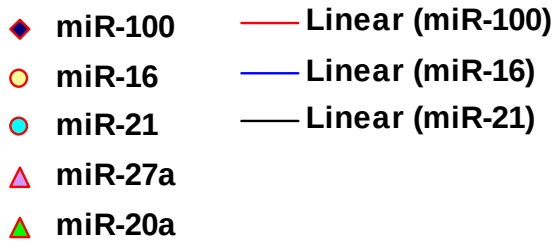


RT in HiFlex Buffer

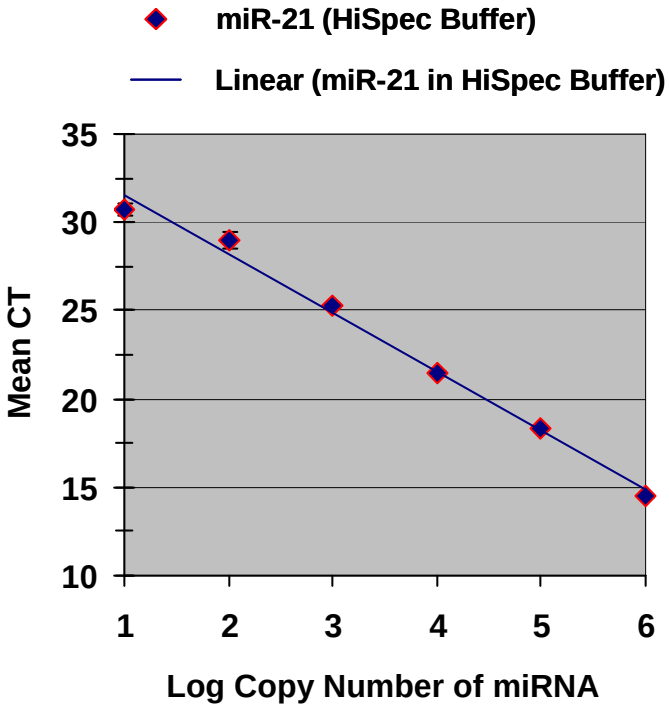


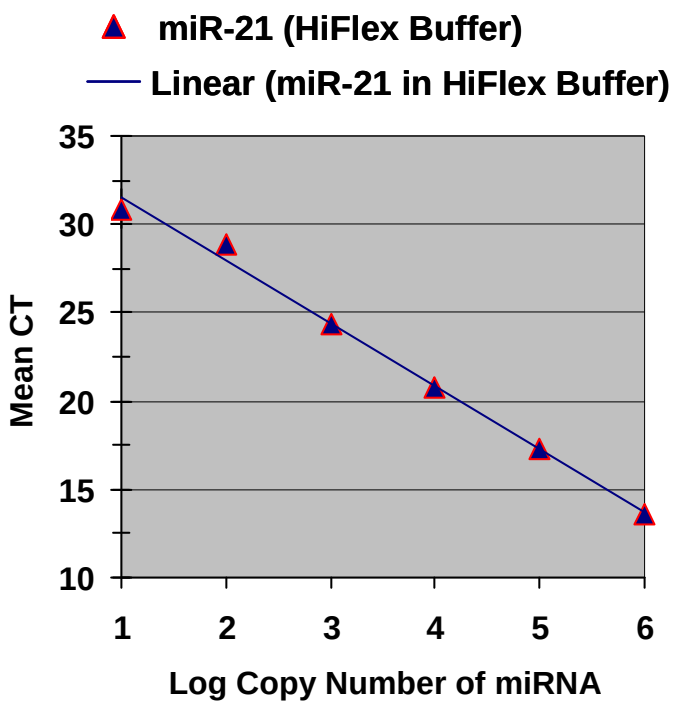
RNA input range (Recommended)

- 5x HiSpec Buffer → 10 pg to 2 µg
- 5x HiFlex Buffer
  - For pre-miRNA detections → max of 500 ng
  - For all the other RNAs →10 pg to 1 µg



High Detection Sensitivity with miScript PCR System: From 10 to 10^6 Copies.





cDNA prepared either in HiSpec Buffer or in HiFlex Buffer using synthetic miR-21 in the presence of a carrier RNA was used as the template.

Real time PCR performed using log dilutions of the cDNA template representing various copy numbers.

When & Why To Use HiSpec Buffer?

- When to use HiSpec Buffer for RT reaction ?
  - cDNA is used exclusively for mature miRNA detection
    - miRNA profiling using miScript miRNome Arrays
    - miScript miRNA PCR Arrays
    - Individual miScript Primer Assays for mature miRNA detection

- Why Use HiSpec Buffer ?
  - Increases sensitivity
  - Eliminates the need to verify the dissociation curves
    - A tedious step in miRNA profiling
  - Higher input amount of RNA tolerated (up to 2 µg of total RNA/RT reaction)
  - Only mature miRNAs & miScript controls are converted into cDNA
  - Any other longer RNA species are not converted into cDNA
    - Hence reduces background noise
    - Higher input amount of RNA tolerated (up to 2 µg of total RNA)
  - All miScript Primer Assays & Arrays are bench validated with cDNA made using HiSpec Buffer

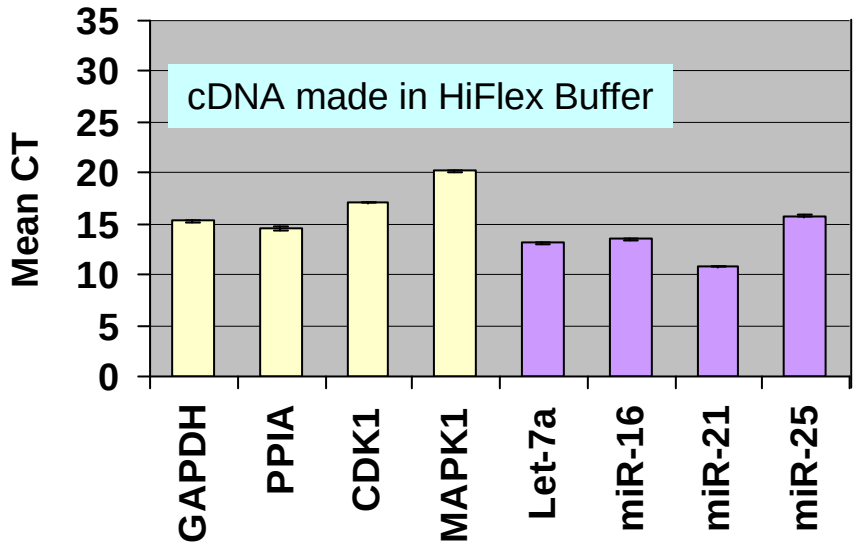
Why To Use HiFlex Buffer?

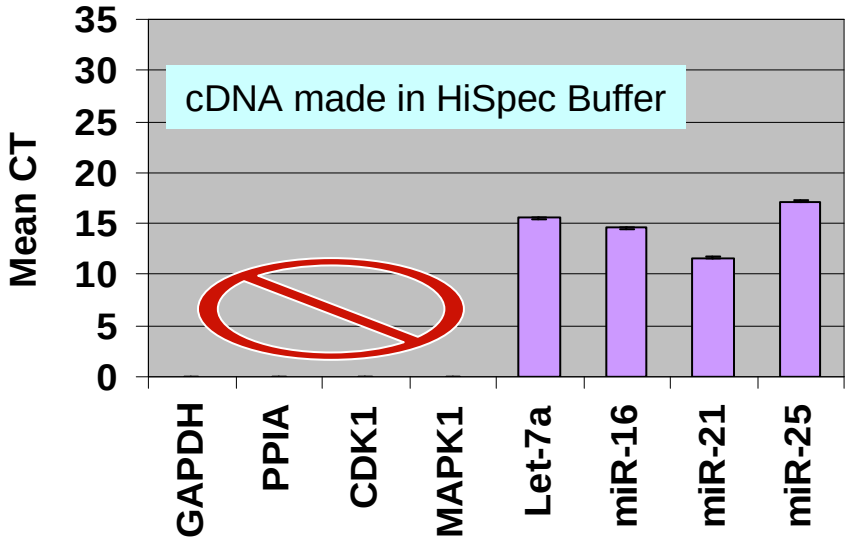
- Why use HiFlex Buffer in RT reaction ?
  - Flexible detection of all RNA molecules is desired
  - Same cDNA can be used for mature miRNAs, Pre-miRNAs, mRNAs & other non coding RNAs
    - Simultaneous detection of mature miRNAs along with its precursors or
    - Simultaneous detection of mature miRNAs along with their mRNA targets
  - Most helpful when starting RNA is a limiting factor when multiple RNA species are to be quantified

HiFlex Buffer:
Detection Of microRNAs & mRNAs From The Same cDNA

HiFlex Buffer should be used to prepare cDNA for the quantification of both mature microRNAs & mRNAs using appropriate Primer Assays.

Long RNAs such as mRNAs are not converted into cDNA during RT reaction in *HiSpec Buffer*



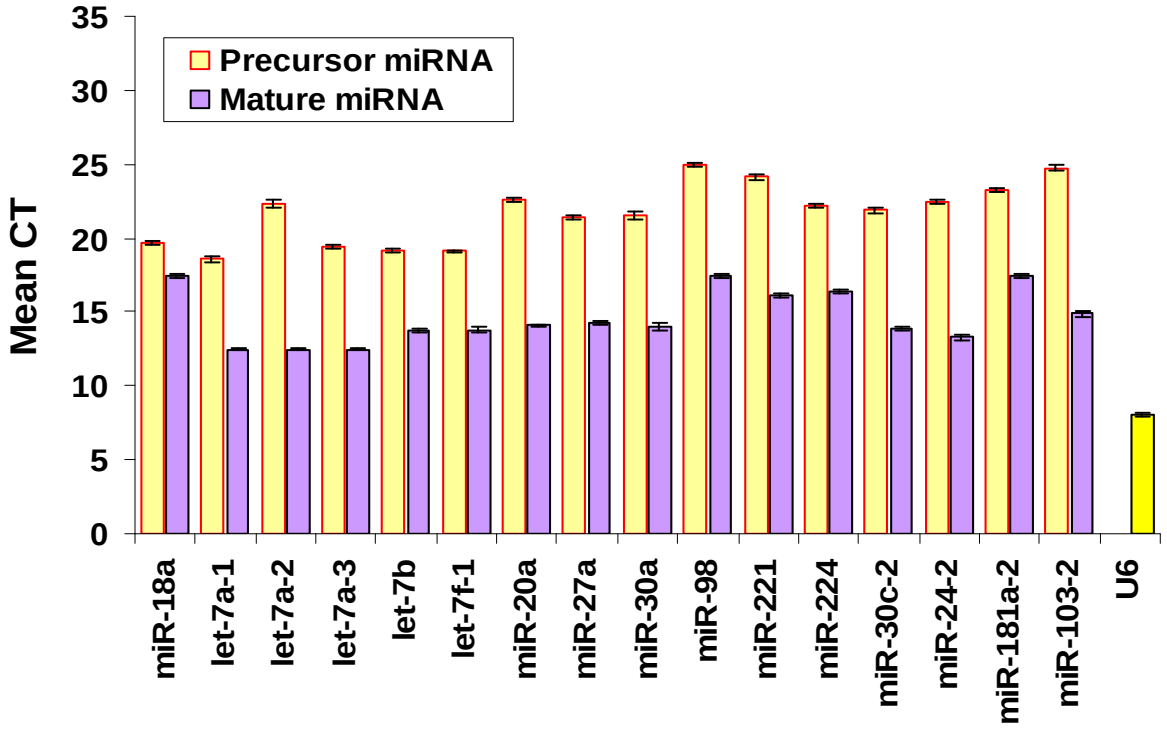


Primer Assays used

- mRNA quantification → QuantiTect Primer Assays
- microRNA quantification → miScript Primer Assays

HiFlex Buffer Allows Parallel Detection of Precursor & Mature miRNAs From The Same cDNA

HiFlex Buffer should be used to prepare cDNA for the quantification of both mature miRNAs and their precursors using appropriate Primer Assays.



Relative levels of various precursor & mature miRNAs in HeLa S3 cells

miScript miRNome PCR Array Content: miRBase v16

Species	Number of Assays (miRBase v16)	Total Number of Plates	
		96 well format	384 well format
Human	**1068**	**12**	**3**
Mouse	**947**	**12**	**3**
Rat	**653**	**8**	**2**
Dog	**277**	**4**	**1**

miScript miRNA Assays & Arrays:

→ Each Assay has been bench validated using synthetic miRNA pool (v16)

miRNA specific FWD Primers only & Universal REV Primer is a part of the miScript SYBR Green PCR Kit

miScript Primer Assays: Highly Specific.

Detection of Let-7 Family of Isoforms With High Specificity

```
CLUSTAL 2.1 multiple sequence alignment

hsa-let-7b    UGAGGUAGUAGGUUGUGUGGUU 22
hsa-let-7c    ----------------A----- 22
hsa-miR-98    ----------A-----A-U--- 22
hsa-let-7d    A--------------CA-A--- 22
hsa-let-7e    --------G-------A-A--- 22
hsa-let-7a    ----------------A-A--- 22
hsa-let-7f    ------------A----A-A--- 22
hsa-let-7g    ------------U----ACA--- 22
hsa-let-7i    ------------U-----CU--- 22
               ******* *   ***     ***
```

% Activity relative to perfect match primer assay as 100%*

cDNA prepared using HiFlex Buffer: cDNA Used in qPCR	Primer Assay Used: Let-7b	Let-7c	miR-98	Let-7d	Let-7e	Let-7a	Let-7f	Let-7g	Let-7i
Let-7b	100.0	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Let-7c	0.5	100.0	0.0	0.0	2.4	0.1	0.0	0.0	0.0
miR-98	0.0	0.2	100.0	0.1	0.0	0.1	0.0	0.0	0.1
Let-7d	0.1	0.0	0.0	100.0	0.0	0.4	0.0	0.0	0.0
Let-7e	0.1	0.0	0.0	0.0	100.0	0.2	0.0	0.0	0.0
Let-7a	0.1	0.6	0.0	0.5	3.1	100.0	0.1	0.0	0.0
Let-7f	0.6	0.1	0.0	0.1	0.0	1.0	100.0	0.1	0.1
Let-7g	0.6	0.2	0.0	0.1	0.0	0.0	0.0	100.0	0.2
Let-7i	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	100.0

*Similar results obtained with cDNA made using HiSpec Buffer

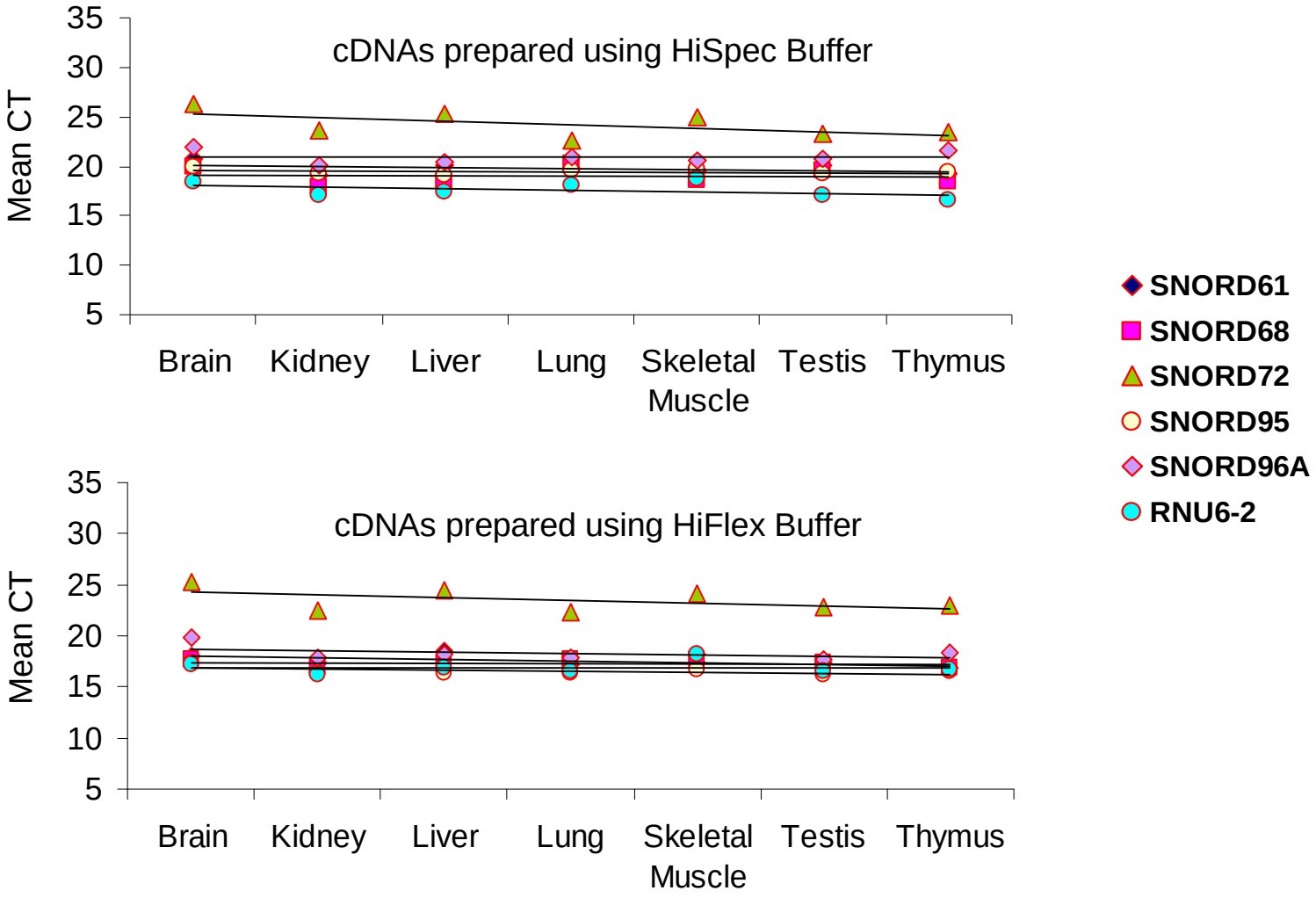
New miScript Controls

- Highly conserved across multiple species
  - Human, Mouse, Rat, dog (and possibly Rhesus, and pig)
  - The same controls work in all these species
- Relatively stable expression in many tissues
- Amplification efficiencies of these assays are 100%
- → Consistent performance in both HiSpec and HiFlex Buffers
- Ideal normalizers for ΔΔCT method of data analysis

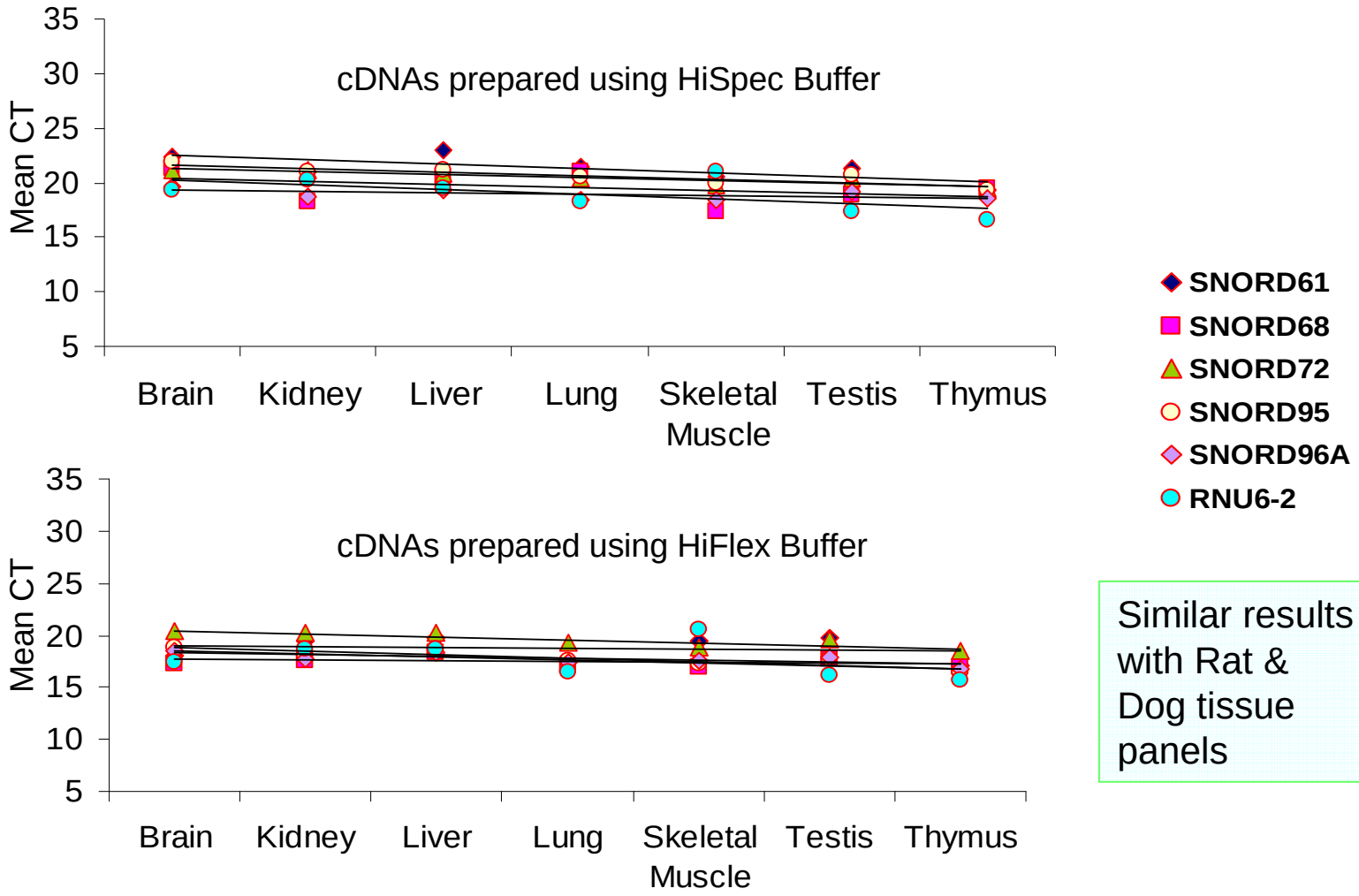
Official Symbol	Gene ID	Accession	Alias	PCR Product Size (bp)
SNORD61	26787	NR_002735	U61; RNU61; HBII-342	83
SNORD68	606500	NR_002450	HBII-202	84
SNORD72	619564	NR_002583	HBII-240	89
SNORD95	619570	NR_002591	U95	81
SNORD96A	619571	NR_002592	U96A	84
RNU6-2	26826	NR_002752	U6; RNU6B	85

MIQE Guidelines recommend the use of multiple Normalization controls

miScript Controls:
Constant Level Of Expression Across A Panel of Human Tissues

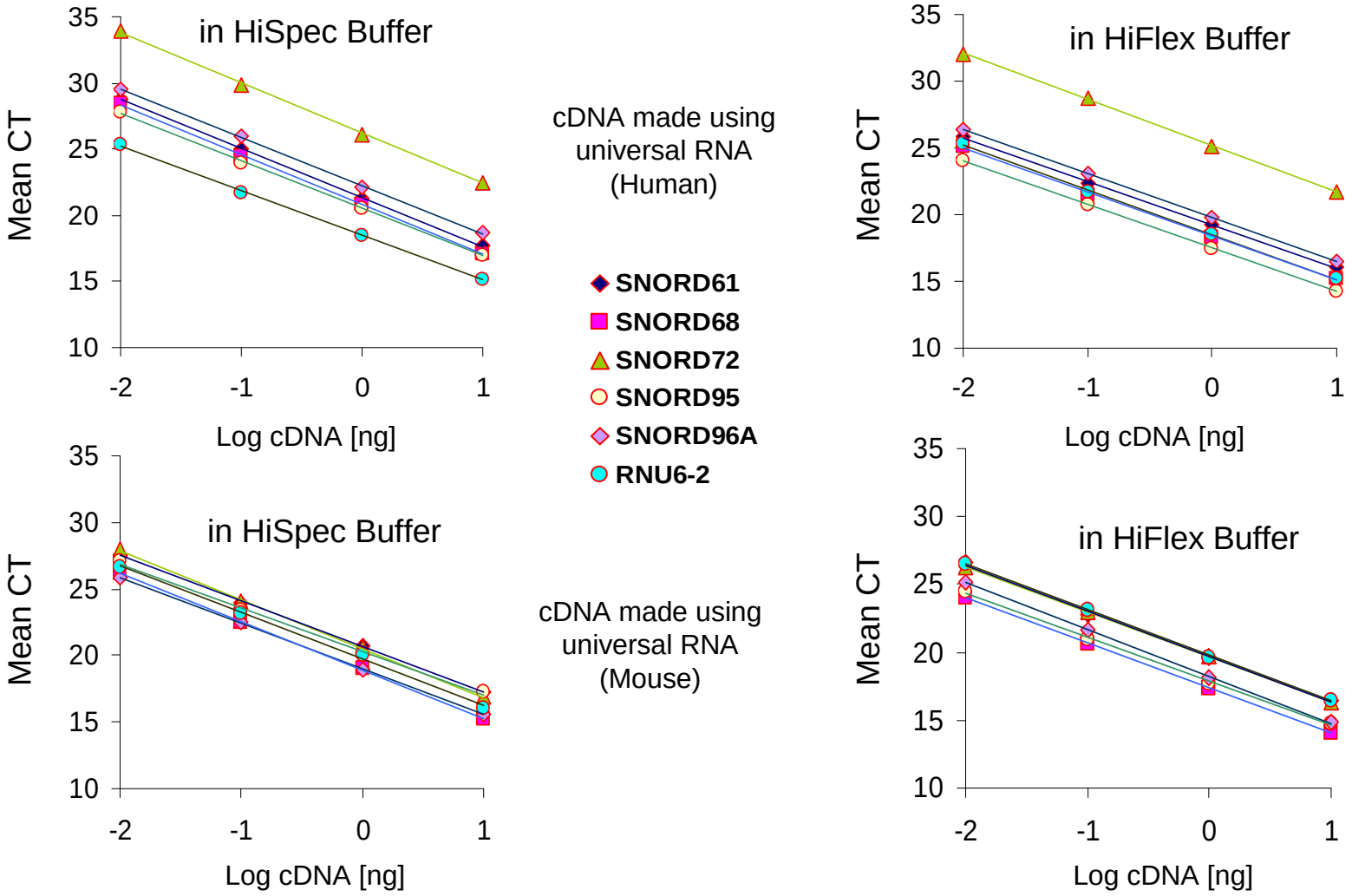


miScript Controls:
Constant Level Of Expression Across A Panel of Mouse Tissues



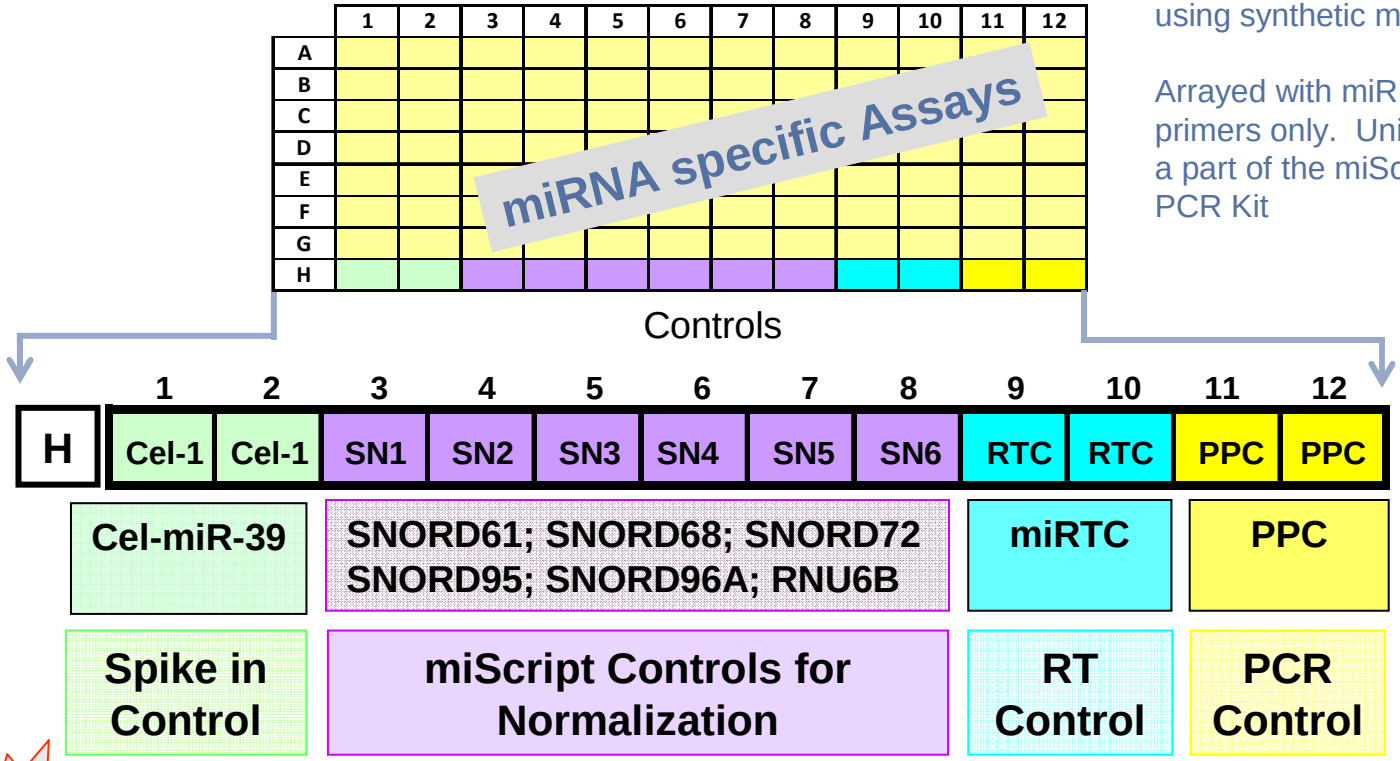
Similar results with Rat & Dog tissue panels

miScript Controls:
Amplification Efficiencies Close To 100%



Similar results with Rat & Dog Universal cDNA

miScript miRNA array format:
Standardized controls (96 well plate)



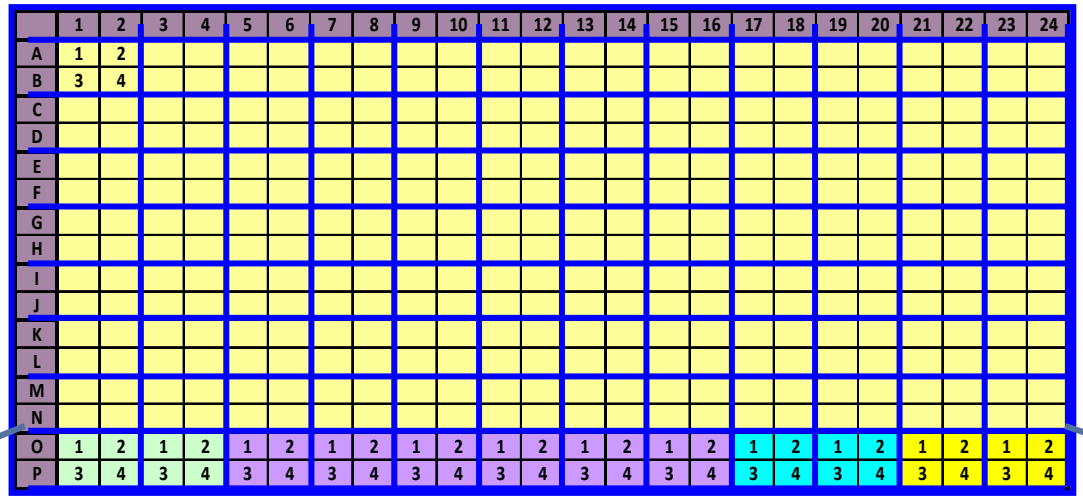
miScript miRNA Assays & Arrays

Each assay has been bench validated using synthetic miRNA pool (v16)

Arrayed with miRNA specific FWD primers only. Universal REV primer is a part of the miScript SYBR Green PCR Kit

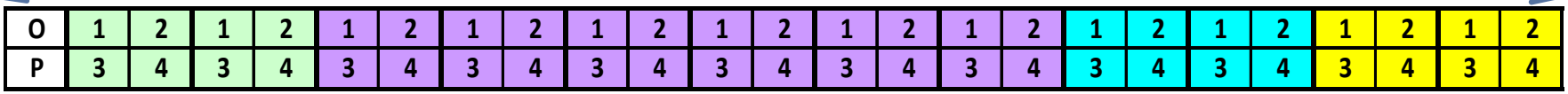
New miScript Controls: Common for Human, Mouse, Rat and Dog Arrays

miScript miRNA array format:
Standardized controls (4X96 well plate)



Arrayed with miRNA specific FWD primer only

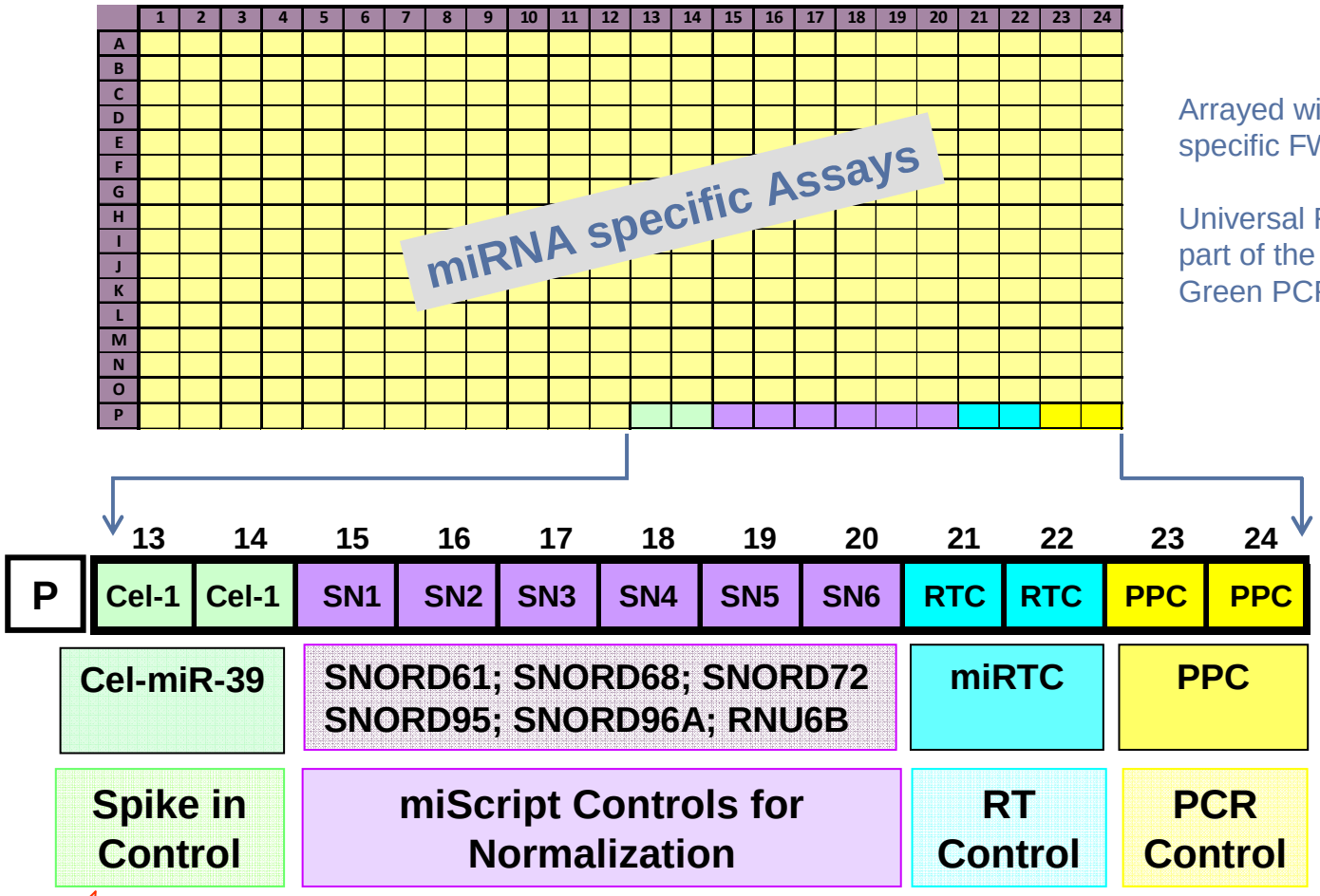
Universal REV primer is a part of the miScript SYBR Green PCR MM



Cel-miR-39	SNORD61; SNORD68; SNORD72 SNORD95; SNORD96A; RNU6B	miRTC	PPC
Spike in Control	miScript Controls for Normalization	RT Control	PCR Control

New miScript Controls: Common for Human, Mouse, Rat and Dog Arrays

miScript miRNA array format:
Standardized controls (384 well plate)



Arrayed with miRNA specific FWD primer only

Universal REV primer is a part of the miScript SYBR Green PCR MM

New miScript Controls: Common for Human, Mouse, Rat and Dog Arrays

miScript miRNA PCR Array portfolio
miRNome, Disease, & Pathway-Focused Analysis




■ miRNome miScript miRNA PCR Arrays (v16)	√	√	√	√
■ Pathway Focused miScript miRNA PCR Arrays:				
□ miFinder Array	√	√	√	√
□ Serum & Plasma	√	√	√	
□ Cancer PathwayFinder Array	√	√	√	
□ Brain Cancer Array	√	√	√	
□ Breast Cancer Array	√	√	√	
□ Ovarian Cancer Array	√	√	√	
□ Cell Development & Differentiation Array	√	√	√	
□ Immunopathology	√	√	√	
□ Inflammatory Response & Autoimmunity Array	√	√	√	
□ Neurological Development & Disease	√	√	√	

QIAGEN Solutions for miRNA Research

Characterizing the miRN*ome* Using the miScript System

miRNA quantification:
Experimental Design & Key Considerations

Scientific question

- A well defined biological question

Experimental sample set

- Sample set containing experimental samples along with appropriate controls
- Statistically meaningful number of replicates (biological replicates)
  - A minimum of 3 replicates recommended
  - Replicates enhance the statistical power
- Inclusion of proper normalization controls & spike in controls in the analysis

Hallmarks of a good experimental system

- Simple & easy to carry out
- High sensitivity & specificity
- Availability of companion research tools

Data Analysis & statistical considerations

- Easy and simple data analysis tools → online data analysis software

Confirmation of the results

- Customizable solutions & single assays

How much RNA is Needed for miRNA Quantification?

Depends on the Detection Limit of the System and miRNA Abundance

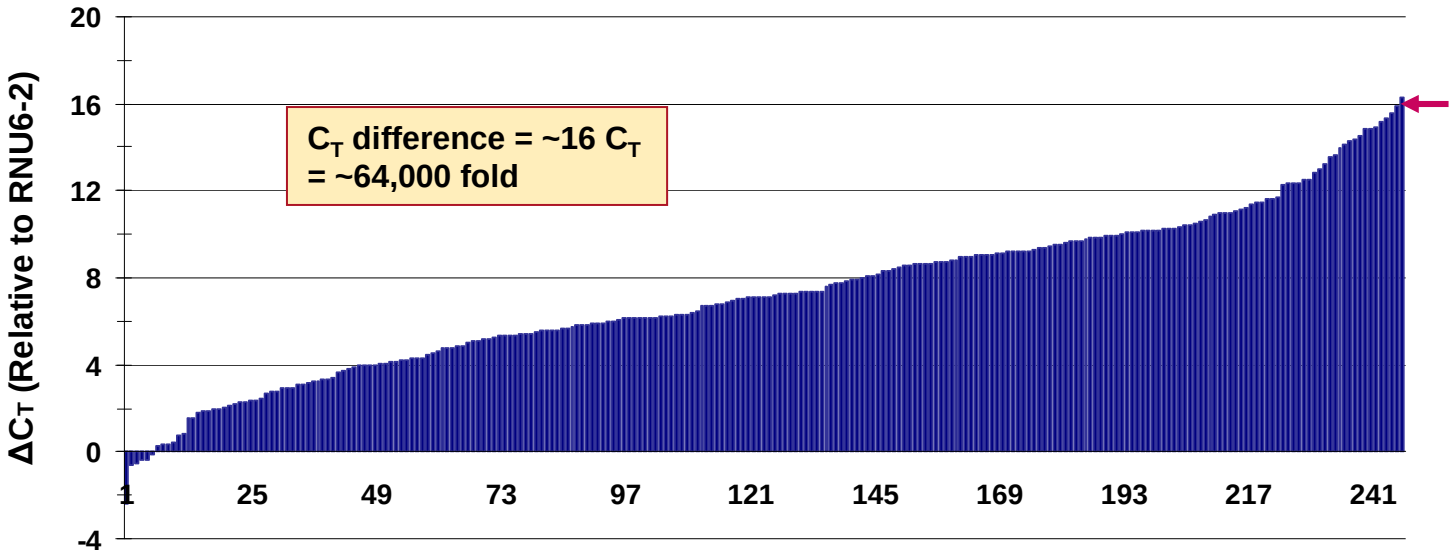
- System sensitivity:
  - miScript RT-PCR can detect 10 copies per PCR
- RNA abundance:
  - A ‘typical’ cell contains ~15-30 pg of total RNA
  - miRNA expression levels vary from as low as 10 copies/cell to as high as 35,000 copies/cell
- How much template is needed to detect an abundant miRNA?
  - An abundant miRNA should be detectable with 10-15 pg of template

How much RNA is Needed for miRNA Quantification?

Depends on the Detection Limit of the System and miRNA Abundance

- What about miRNAs expressed at ~10-100 copies/cell?
  - Considering that 10x more template result in a 3.3 CT shift
  - Use 10-20 times more template (0.1 ng-0.2 ng) to detect rare miRNAs
- To be able to 'call with certainty' a miRNA is 'not expressed'
  - use >20x more RNA (0.5 to 1.5 ng)
- As a guideline, consider using ~ 50 pg to 500 pg template/PCR
  - Up to 20,000 PCR reactions per 1 µg miScript RT reaction
  - Use an RT system that can work in a broad input RNA range.
    - miScript allows 10 pg - 1µg in put RNA into RT reaction

miRNA Expression In HeLa Cells

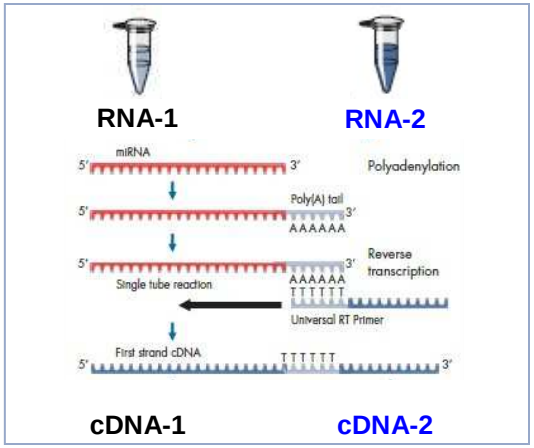


Deep sequencing results show that miRNA reads range from 20 to 292,000 (in *HeLa* cells)

Shin, C., et. al. 2010; *Mol. Cell, 38, 789*

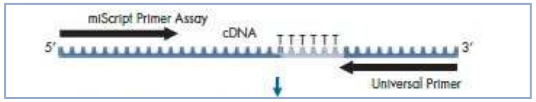
miScript miRNA PCR Array workflow:
Genome-wide, disease, & pathway-focused analysis

1. Convert miRNA into cDNA

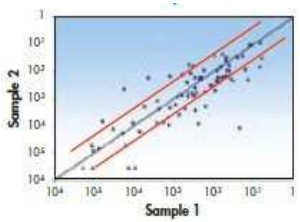


2. Assemble Real time PCR

3. Run in real-time PCR cycler

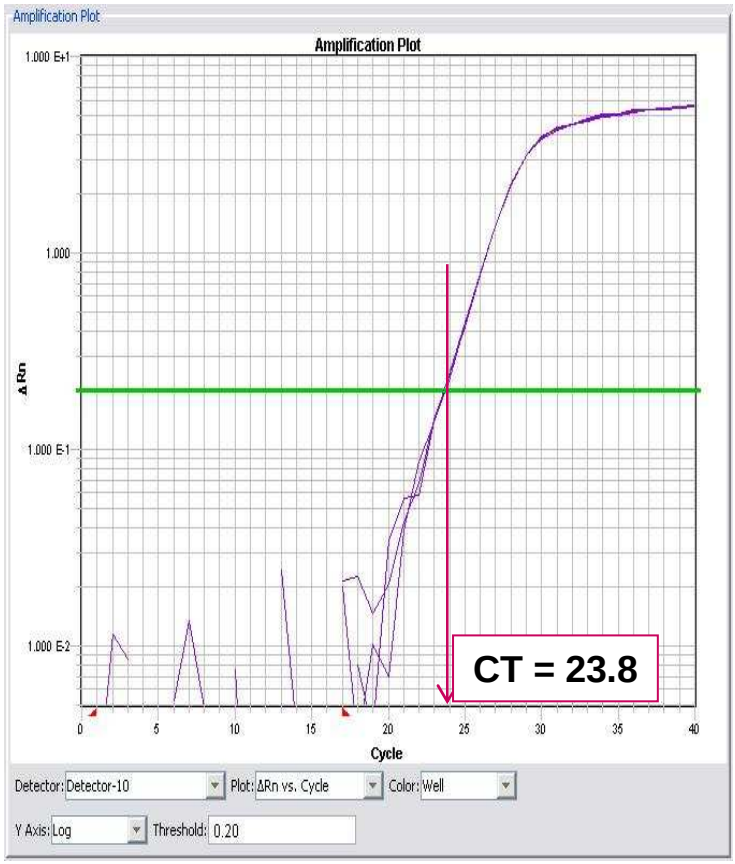


4. Data Analysis



- cDNA Synthesis in HiSpec Buffer
  - **1** hour
- Load Plates
  - 2 minutes
- Run 40 cycle qPCR Program
  - 2 hours
- Upload and analyze Data
  - 15 minutes

qRT-PCR Data Analysis



- Absolute quantification
  - Absolute input copies, based on a standard curve
- Relative quantification
  - Comparative CT method
    (also known as the $2^{-\Delta\Delta CT}$ method)
  - Selection of internal control
  - Selection of calibrator
    (e.g. untreated control or normal sample)
  - Assumes that the PCR efficiency of the target gene is similar to the internal control gene (and that the efficiency of the PCR is close to 1)
  - Fold change = $2^{-\Delta\Delta CT}$

= [{gene of interest (CT) – internal control (CT) sample A} –
{gene of interest (CT) - internal control (CT) sample B}]

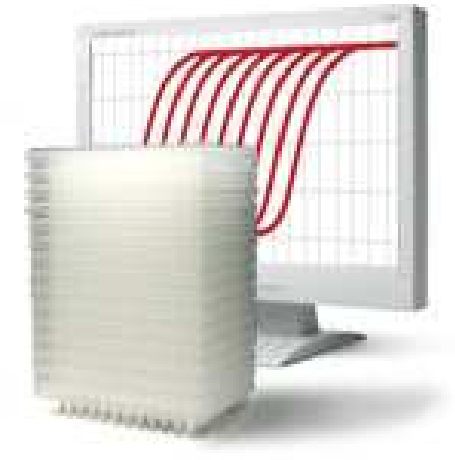
(1) Schmittgen TD, Livak KJ.(2008):Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc.;3(6):1101-8

(2) Livak, KJ, and Schmittgen, TD.(2001): Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method METHODS 25, 402–408

(3) www.Gene-Quantification.info

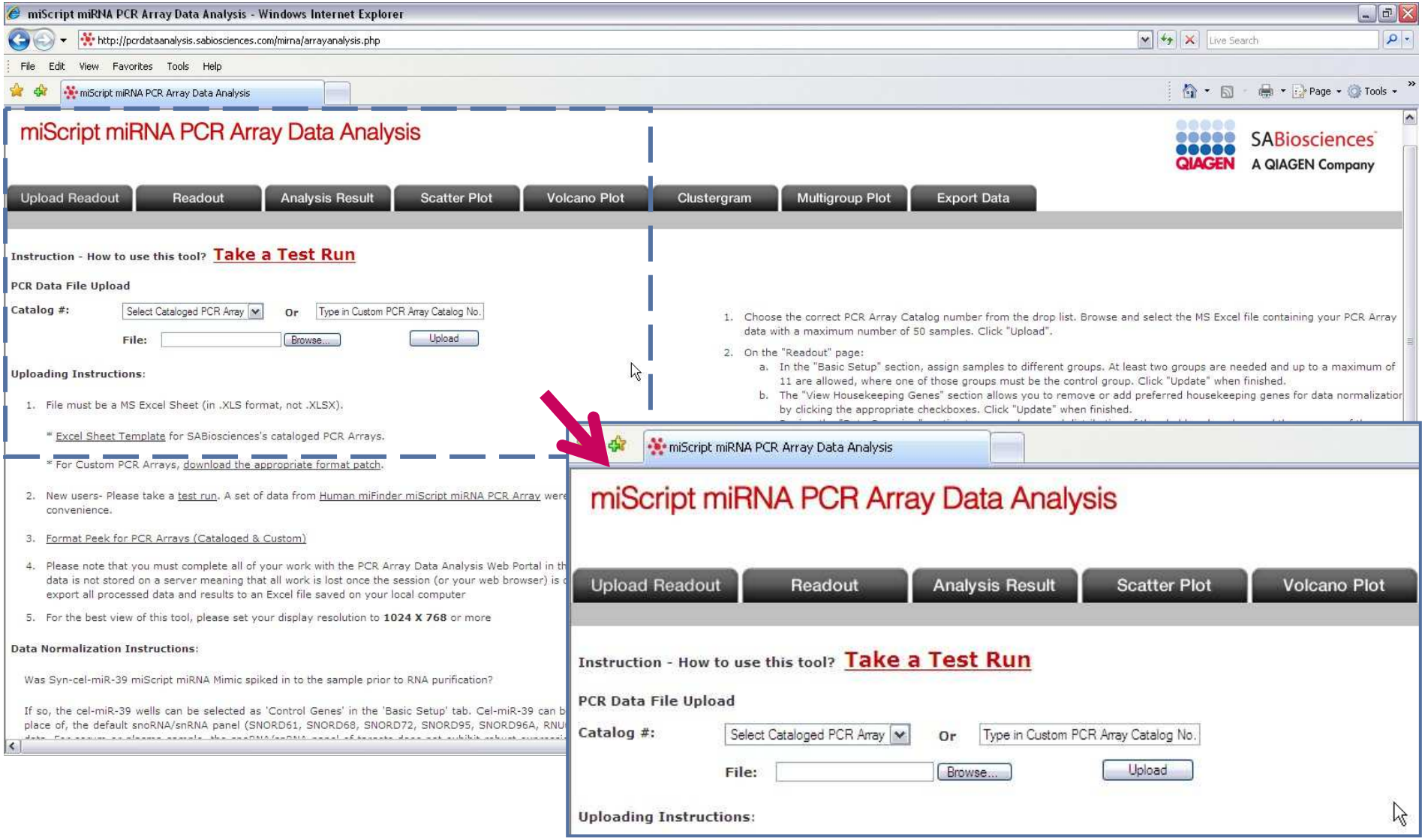
miScript miRNA PCR Array Data Analysis tool

- Web-Based Software
  - No installation needed
- From Raw Ct Values to Fold Change Results
  - Using ΔΔC(t) Method
- Multiple Analysis Formats
  - Scatter Plot
  - Volcano Plot
  - Multi-Group Plot
  - Clustergram

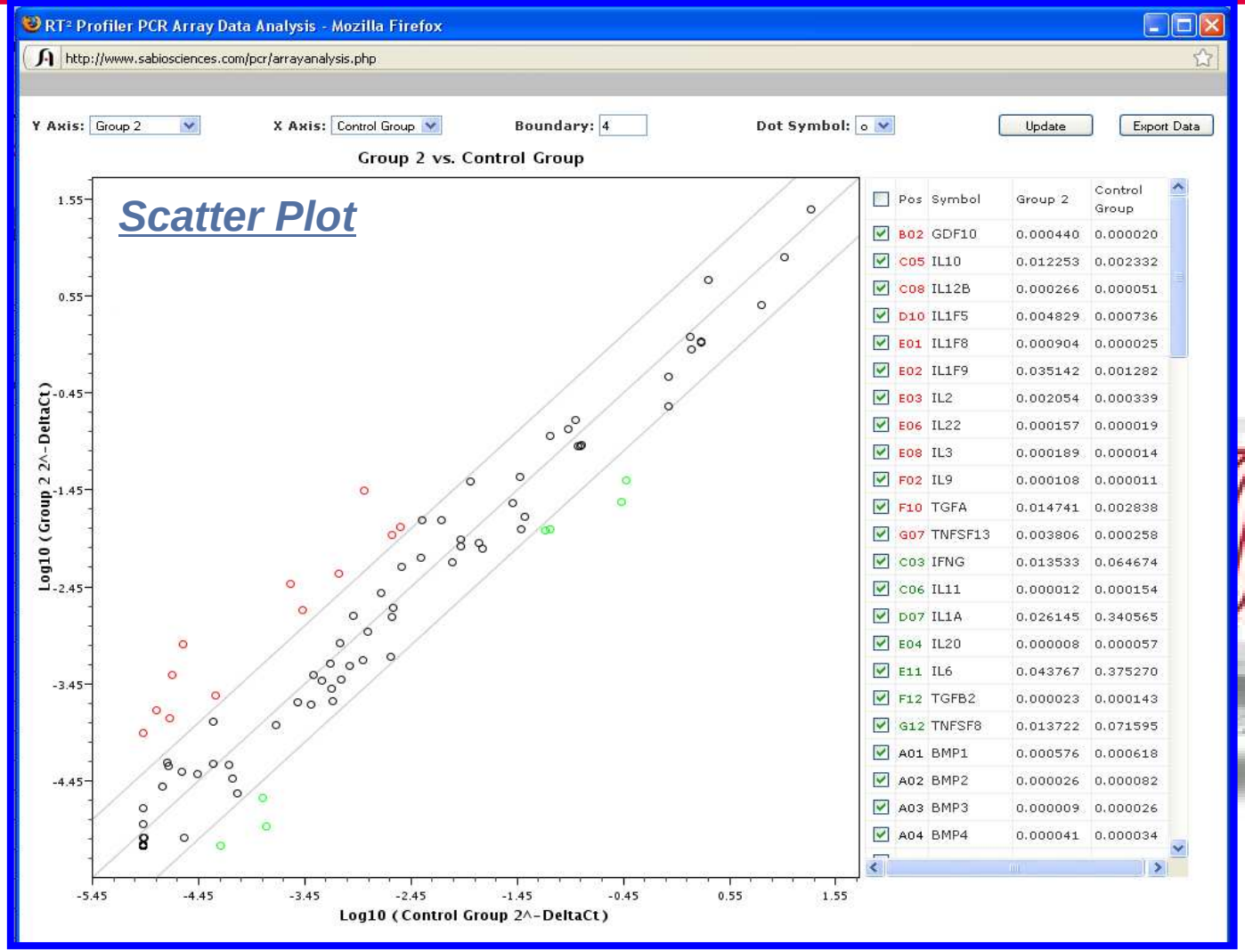


Also available as downloadable Excel templates

Free Online Data Analysis tool: Data Analysis Made Easy & Simple

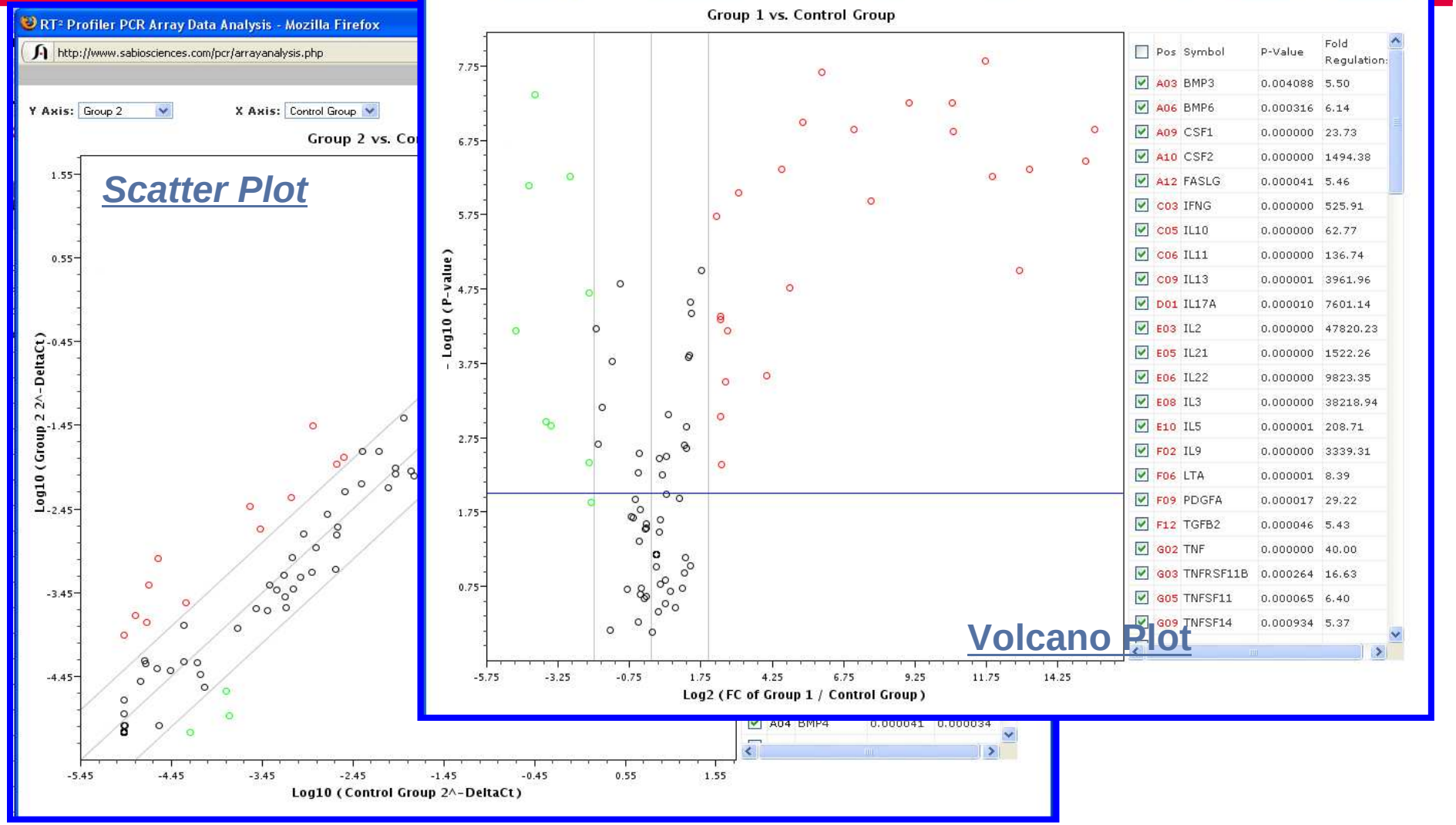


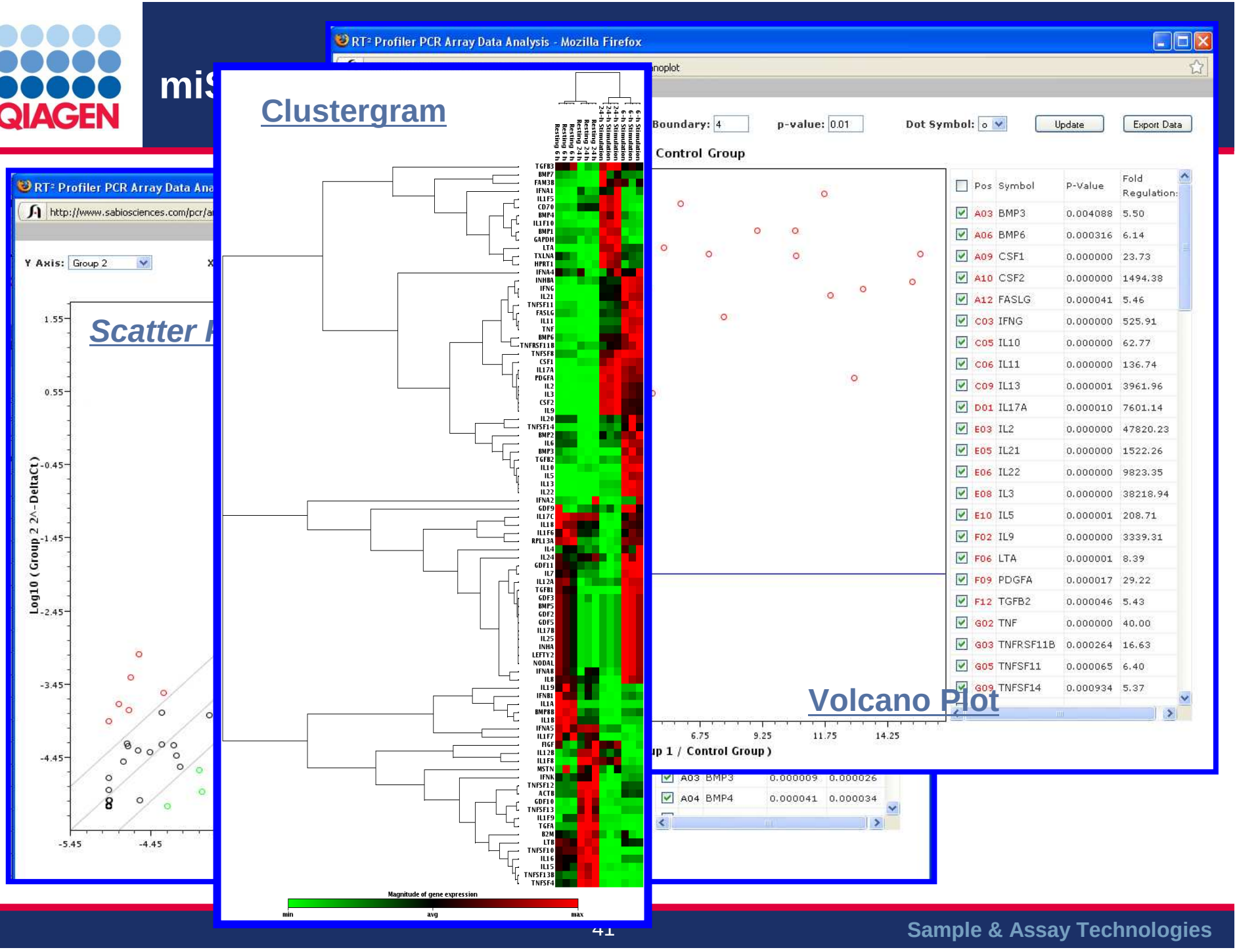
miScript miRNA PCR Array Data Analysis



Pos	Symbol	Group 2	Control Group
B02	GDF10	0.000440	0.000020
C05	IL10	0.012253	0.002332
C08	IL12B	0.000266	0.000051
D10	IL1F5	0.004829	0.000736
E01	IL1F8	0.000904	0.000025
E02	IL1F9	0.035142	0.001282
E03	IL2	0.002054	0.000339
E06	IL22	0.000157	0.000019
E08	IL3	0.000189	0.000014
F02	IL9	0.000108	0.000011
F10	TGFA	0.014741	0.002838
G07	TNFSF13	0.003806	0.000258
C03	IFNG	0.013533	0.064674
C06	IL11	0.000012	0.000154
D07	IL1A	0.026145	0.340565
E04	IL20	0.000008	0.000057
E11	IL6	0.043767	0.375270
F12	TGFB2	0.000023	0.000143
G12	TNFSF8	0.013722	0.071595
A01	BMP1	0.000576	0.000618
A02	BMP2	0.000026	0.000082
A03	BMP3	0.000009	0.000026
A04	BMP4	0.000041	0.000034

miScript m


RT² Profiler PCR Array Data Analysis - Mozilla Firefox
http://www.sabiosciences.com/pcr/arrayanalysis.php
Y Axis: Group 2
X Axis: Control Group
Group 2 vs. Co
Scatter Plot
Log10 (Group 2 2^-DeltaCt)
Log10 (Control Group 2^-DeltaCt)
RT² Profiler PCR Array Data Analysis - Mozilla Firefox
http://www.sabiosciences.com/pcr/arrayanalysis.php?target=volcanoplot
Treated: Group 1
Control: Control Group
Boundary: 4
p-value: 0.01
Dot Symbol: o
Update
Export Data
Group 1 vs. Control Group
- Log10 (P-value)
Log2 (FC of Group 1 / Control Group)
Pos Symbol P-Value Fold Regulation
A03 BMP3 0.004088 5.50
A06 BMP6 0.000316 6.14
A09 CSF1 0.000000 23.73
A10 CSF2 0.000000 1494.38
A12 FASLG 0.000041 5.46
C03 IFNG 0.000000 525.91
C05 IL10 0.000000 62.77
C06 IL11 0.000000 136.74
C09 IL13 0.000001 3961.96
D01 IL17A 0.000010 7601.14
E03 IL2 0.000000 47820.23
E05 IL21 0.000000 1522.26
E06 IL22 0.000000 9823.35
E08 IL3 0.000000 38218.94
E10 IL5 0.000001 208.71
F02 IL9 0.000000 3339.31
F06 LTA 0.000001 8.39
F09 PDGFA 0.000017 29.22
F12 TGFB2 0.000046 5.43
G02 TNF 0.000000 40.00
G03 TNFRSF11B 0.000264 16.63
G05 TNFSF11 0.000065 6.40
G09 TNFSF14 0.000934 5.37
Volcano Plot

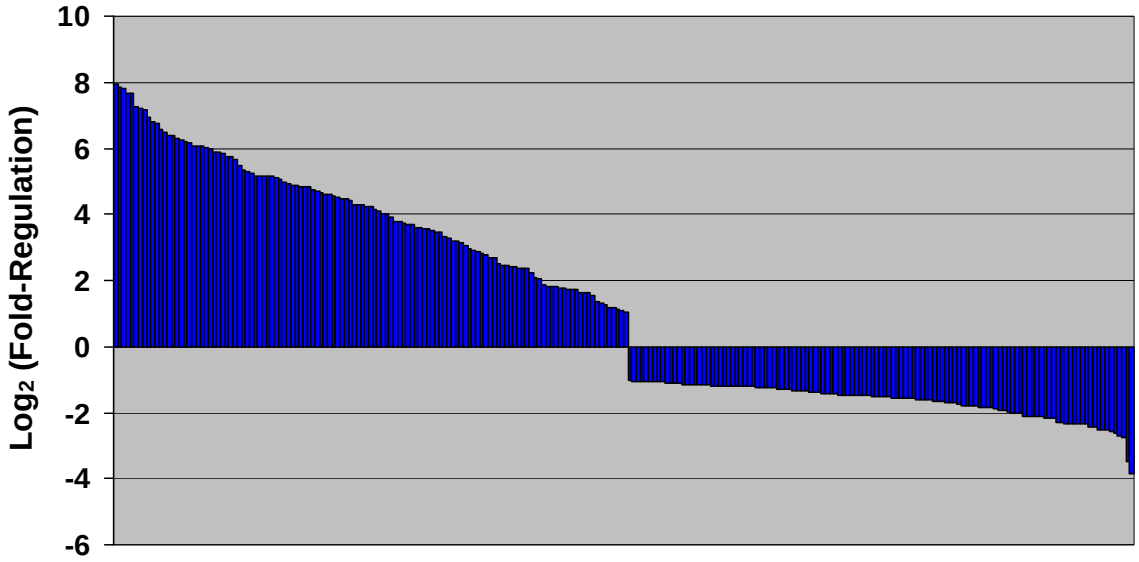

RT² Profiler PCR Array Data Analysis - Mozilla Firefox
Clustergram
Scatter Plot
Volcano Plot
Boundary: 4
p-value: 0.01
Dot Symbol:
Update
Export Data
Control Group
Y Axis: Group 2
Log10 (Group 2 2^-DeltaCt)
Magnitude of gene expression
min
avg
max
Pos Symbol P-Value Fold Regulation
A03 BMP3 0.004088 5.50
A06 BMP6 0.000316 6.14
A09 CSF1 0.000000 23.73
A10 CSF2 0.000000 1494.38
A12 FASLG 0.000041 5.46
C03 IFNG 0.000000 525.91
C05 IL10 0.000000 62.77
C06 IL11 0.000000 136.74
C09 IL13 0.000001 3961.96
D01 IL17A 0.000010 7601.14
E03 IL2 0.000000 47820.23
E05 IL21 0.000000 1522.26
E06 IL22 0.000000 9823.35
E08 IL3 0.000000 38218.94
E10 IL5 0.000001 208.71
F02 IL9 0.000000 3339.31
F06 LTA 0.000001 8.39
F09 PDGFA 0.000017 29.22
F12 TGFB2 0.000046 5.43
G02 TNF 0.000000 40.00
G03 TNFRSF11B 0.000264 16.63
G05 TNFSF11 0.000065 6.40
G09 TNFSF14 0.000934 5.37

miRNA profiling of HCT 116 Colorectal Cancer Cells:
Following treatment with 5-aza-2'-deoxycytidine (AZDC)

- 5-aza-2'-deoxycytidine (AZDC) is an irreversible inhibitor of DNA methylation.
- AZDC incorporates into DNA & covalently bind to the active site of the DNMT
- HCT116 cells were treated with AZDC, and Untreated cells were used as controls
- Total RNA from each was used to make cDNA with miScript II RT in HiSpec Buffer
- Human whole-miRNome miScript miRNA PCR Array set was used to profile of mature miRNA expression
- Data analysis was performed using the online miScript miRNA PCR Array data analysis tool.

DNMT: DNA Methyl Transferase enzyme

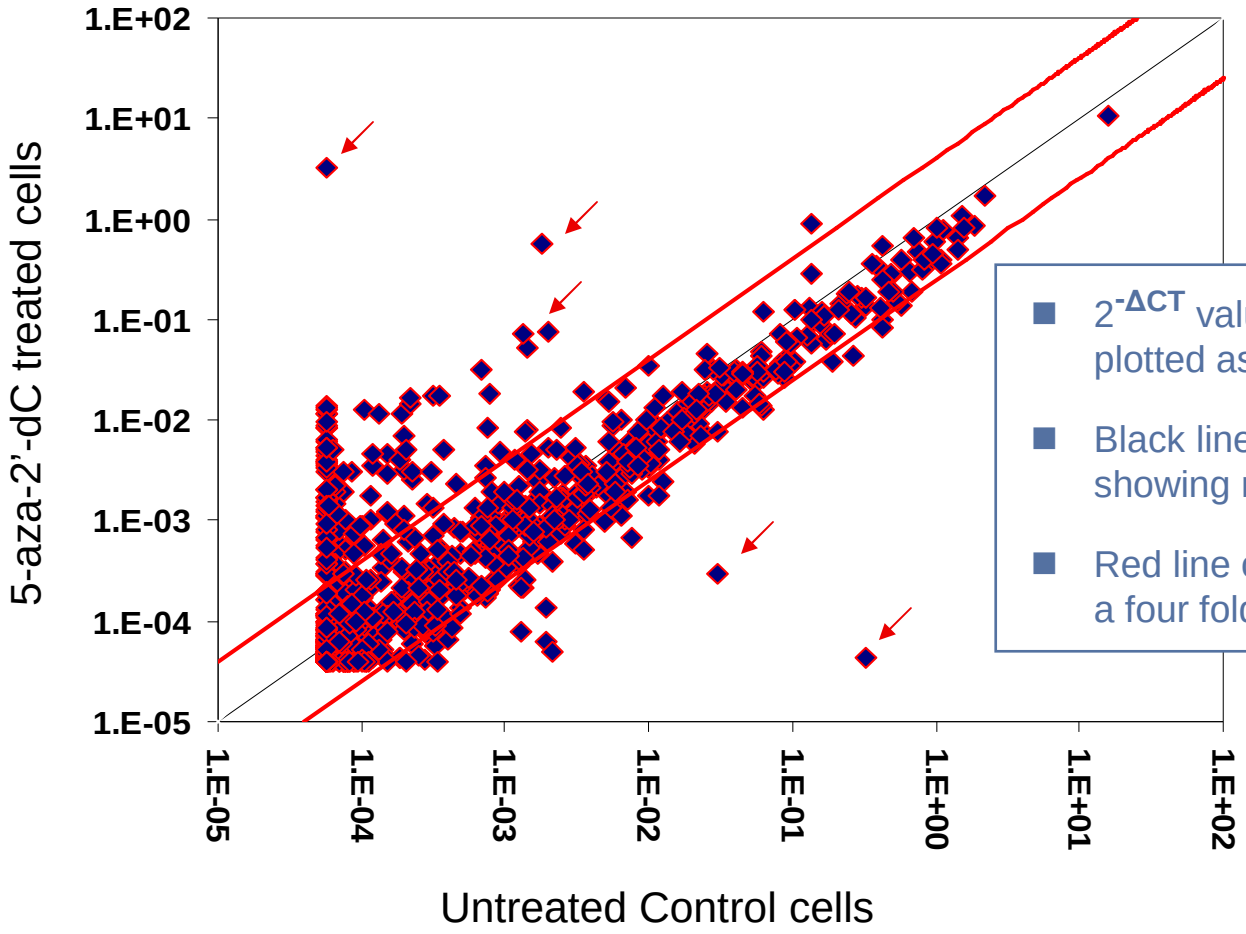
miRNA profiling of HCT 116 Colorectal Cancer Cells:
Following treatment with 5-aza-2'-deoxycytidine (AZDC)



>2 fold UP:	250 miRNAs
>10 fold UP:	164 miRNAs
>2 fold DOWN:	369 miRNAs
>10 fold DOWN:	51 miRNAs

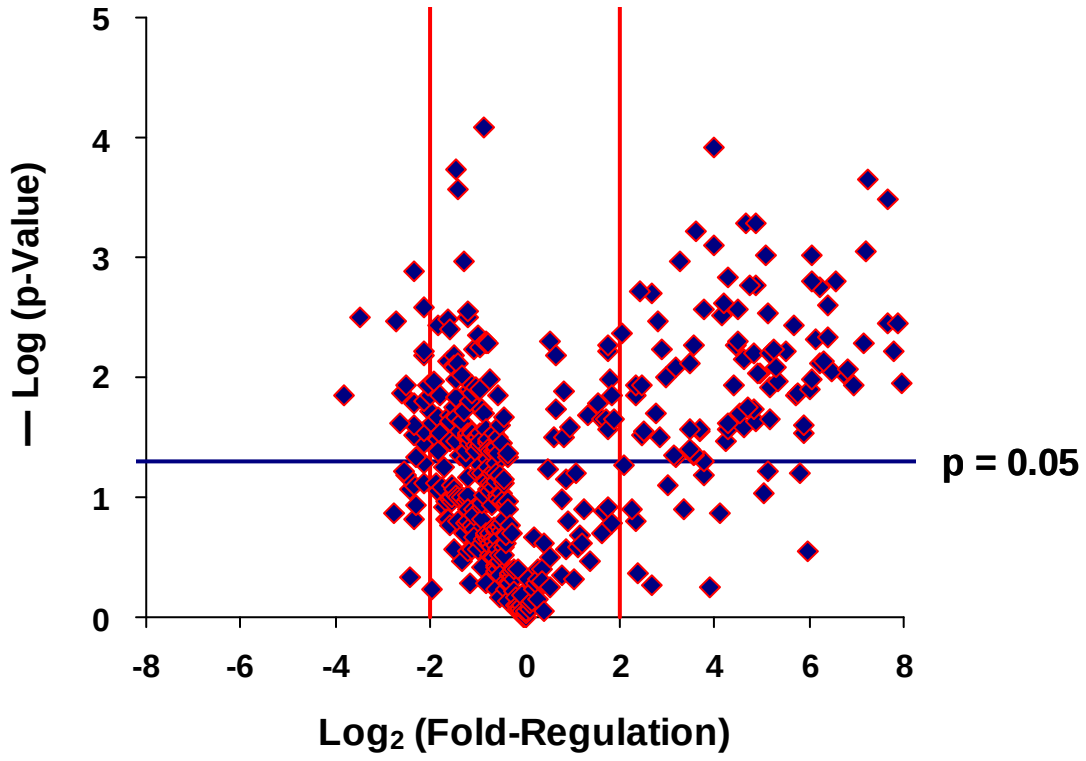
miRNA up regulation likely due to demethylation associated promoter activation

Scatter Plot:
AZDC Treated HCT 116 Colorectal Cancer Cells

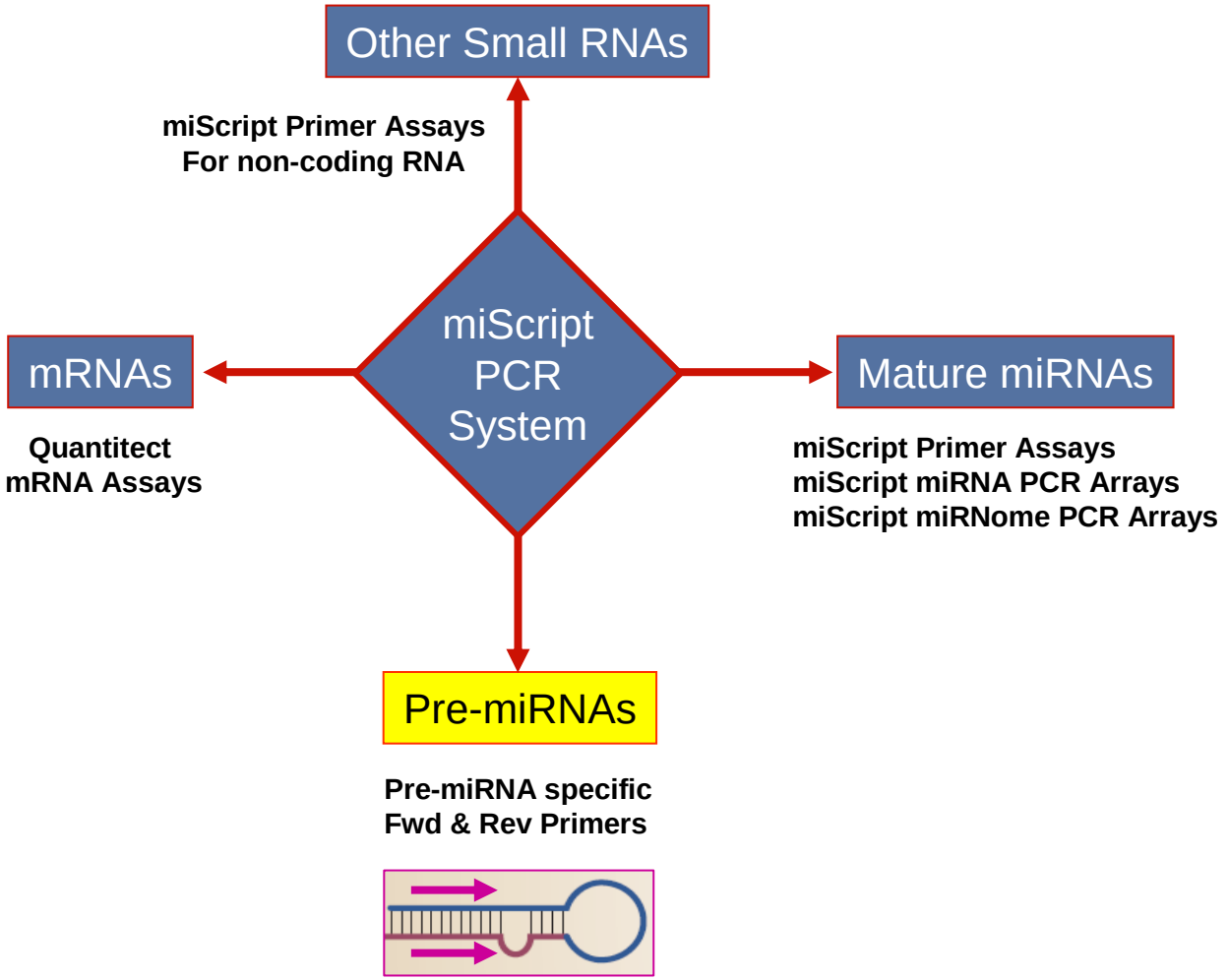


- $2^{-\Delta CT}$ values of each sample were plotted as scattered plot
- Black line represents miRNAs showing no regulation
- Red line on either side represents a four fold UP or DOWN regulation

Volcano Plot:
AZDC Treated HCT 116 Colorectal Cancer Cells



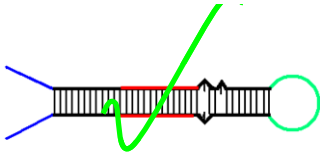
miScript PCR System: Pre-miRNA Quantification



Pre-miRNA Stem-Loop Quantification

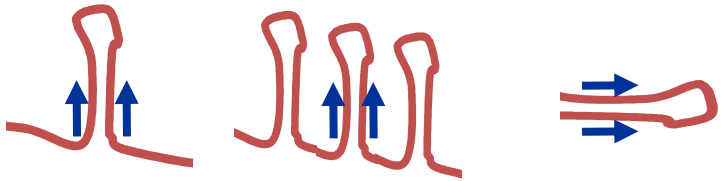
- Study transcriptional and/or post-transcriptional regulation in miRNA biogenesis
- Definitively identify which closely related genomic loci are expressed

miScript Precursor Assays



- Precursor specific FWD & REV primer
- Specific for precursor miRNAs only

- Precursor assays detect all RNA species that contain the specific stem loop



Pri-miRNAs Pre-miRNAs

Expression of Let-7a precursors in various tissues

CHR-9, Intergenic

Homo sapiens let-7a-1 stem-loop

```
     u   gu                    uuagggucacac
uggga gag  aguagguuguauaguu              c
|||||| |||  ||||||||||||||||              c
aucou uuc  ucaucuaacauaucaa              a
     -   ug                    uagagggucacc
```

CHR-11, NR_024430.1: mir-100-let-7a-2 cluster host gene (non-protein coding)

Homo sapiens let-7a-2 stem-loop

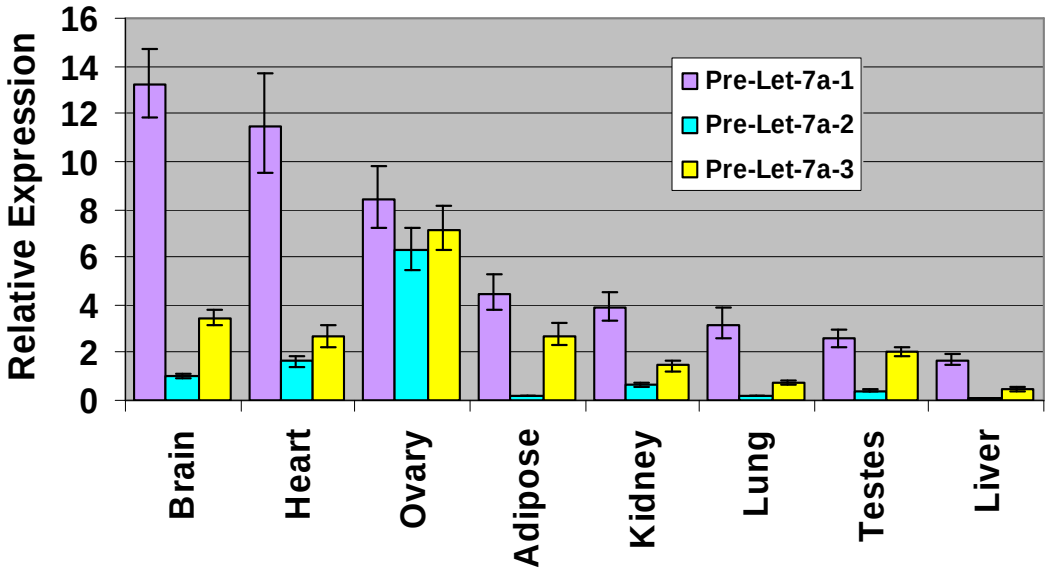
```
   uu   g   u                 uagaa ua a
agg  gag uag agguuguauaguu        u  c u
|||  ||| ||| ||||||||||||||       |  | c
ucc  uuc auc uccgacaugucaa        a  g a
  -u   g   c                 --uag gg a
```

CHR-22, NR_027033.1: MIRLET7B host gene (non-protein coding)

Homo sapiens let-7a-3 stem-loop

```
   u   gu                 ---------
ggg gag  aguagguuguauaguu          uggggcu
||| |||  ||||||||||||||||          |||||| c
ucc uuc  ucaucuaacauaucaa          gucccgu
   u   ug                 uaggguauc
```

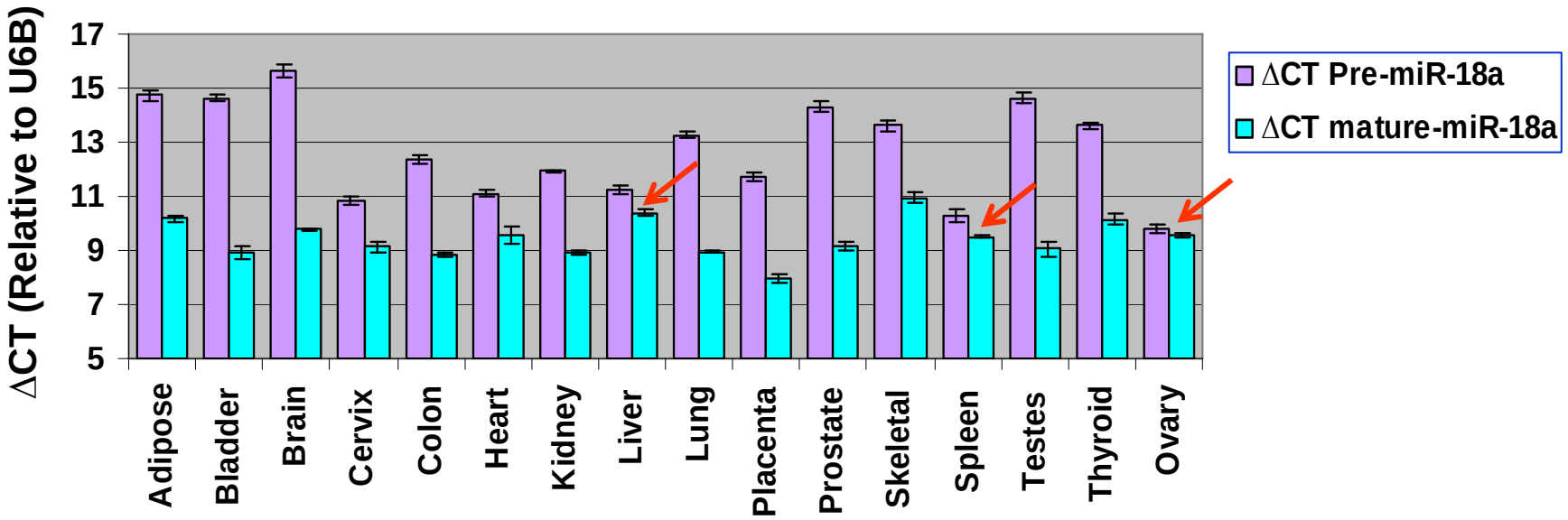
Mature hsa-Let-7a**:** 5'-UGAGGUAGUAGGUUGUAUAGUU-3'

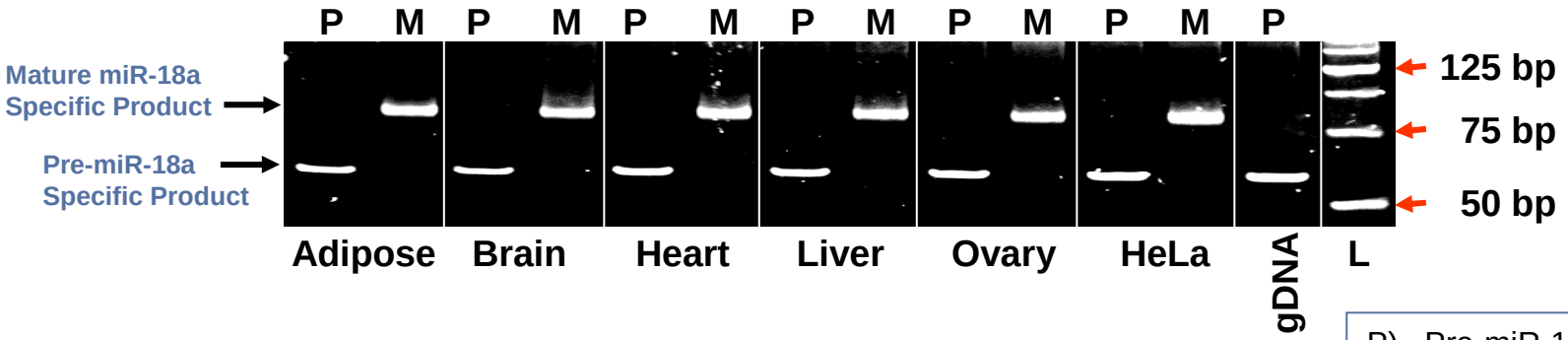


Relative Expression = $2^{-\Delta CT}$ X 10^5
Normalizer Used: U6B
10 ng cDNA /PCR

Expression of miR-18a and its Precursor

A member of the miR-17-92 Cluster (13q31.3)





P) Pre-miR-18a specific
M) Mature miR-18a specific

Summary

- Challenges in miRNA quantification
- miRNA quantification by real time PCR
- Available tools for miRNA profiling experiments
- Critical aspects about template amount requirements
- Examples of miRNA profiling studies
- Precursor detection
  - Measuring the relative levels of mature miRNA and its precursor
  - Identifying the genetic locus responsible for a given miRNA expression in a cell
- miScript miRNA arrays for simple and easy miRNA Profiling
- Data analysis

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- Cat. No. (e.g., SI00299551, QT01342509)
- Sanger ID or Accession (e.g., hsa-let-7b, MI0000063)

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Species: All

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Nematode (Caenorhabditis elegans)
Zebrafish (Danio rerio)
Frog (clawed frog) (Xenopus laevis)
Cow (Bos taurus)
Epstein Barr virus
Human cytomegalovirus
Human immunodeficiency virus 1
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Mareks disease virus type 2
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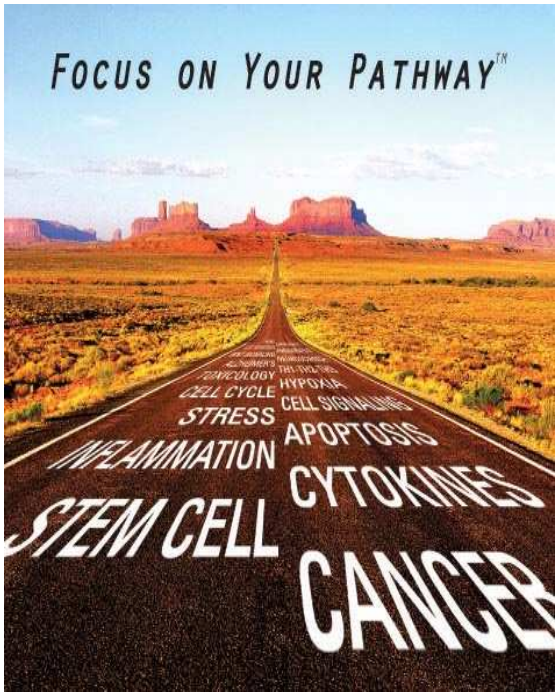

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Questions

Webinar 3:	Profiling miRNA Expression: On the Road To Biomarker Development
Date:	October 19, 2011, 1:00 pm EST
Speaker:	Eric Lader Ph. D. Senior Director, QIAGEN R&D

Thank you!

Subu Yerramilli
Subu.yerramilli@qiagen.com