

miRNA/mRNA Expression Profiling From Single ES Cells

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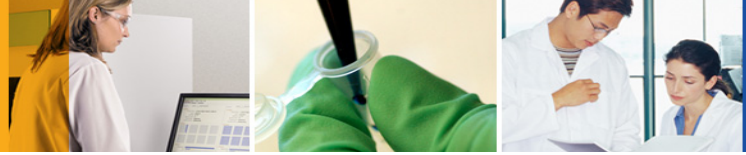
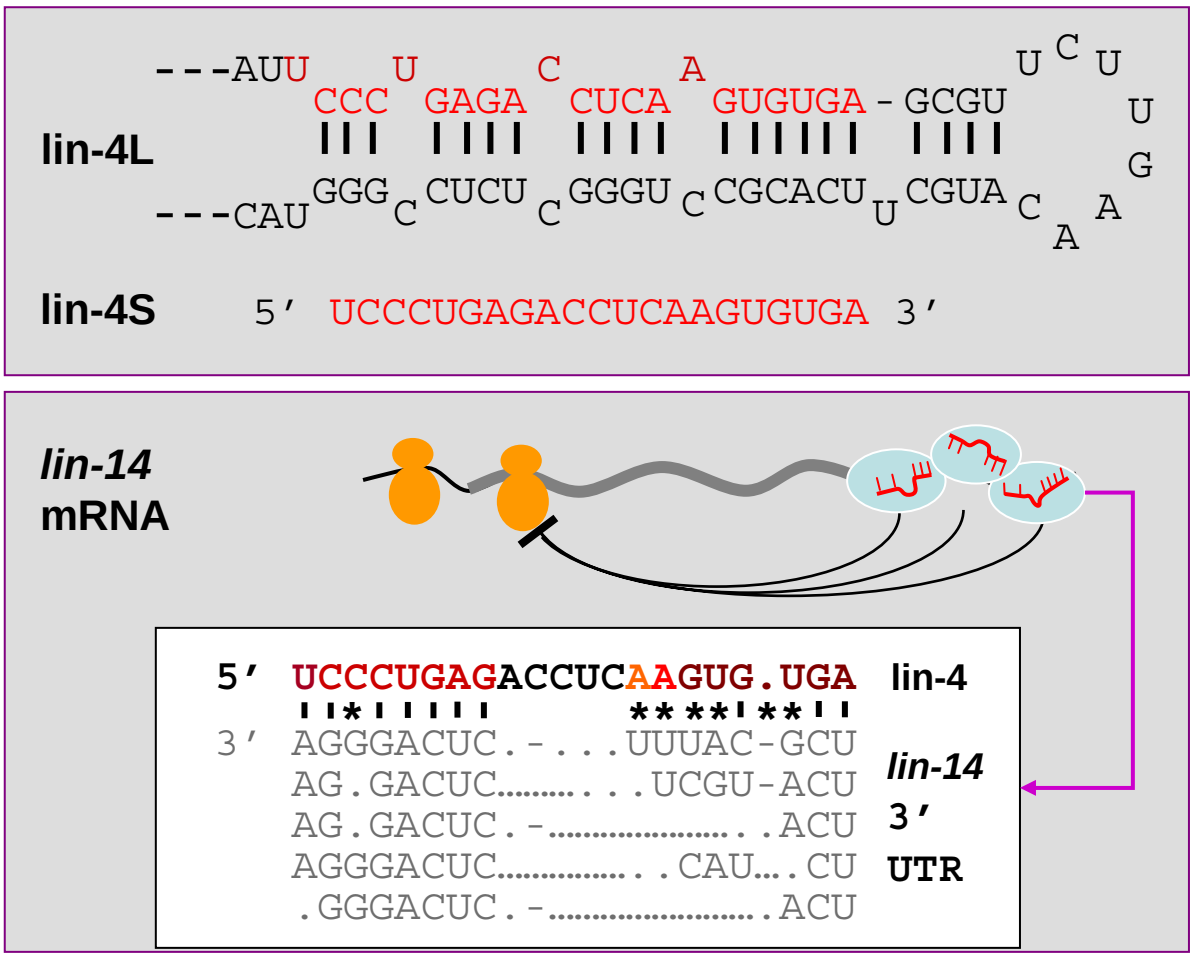
Applied Biosystems



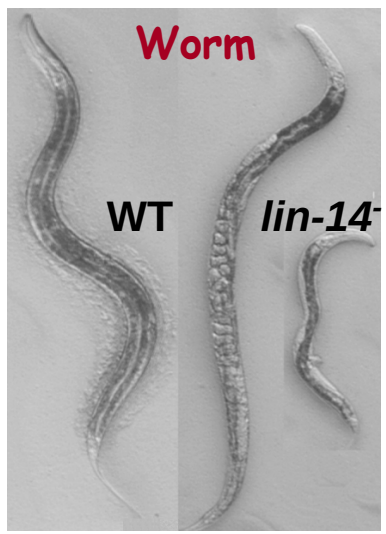
Why do we need single cell expression analysis?

- Limited biological samples or RNA
  - LCM
  - Saliva and plasma
- Single cell biology
  - Development of embryos and neurons etc.
- Samples with complex cell types
  - Tumors etc.
- Pharmacogenomics
  - A clearer understanding of gene expression differences at single cell level provides an opportunity to develop drugs with more targeted pharmacologies or with decreased side effects.
- ES cell QC
  - Evaluate the degree of heterogeneity

Discovery of microRNAs: Tiny *lin-4* story



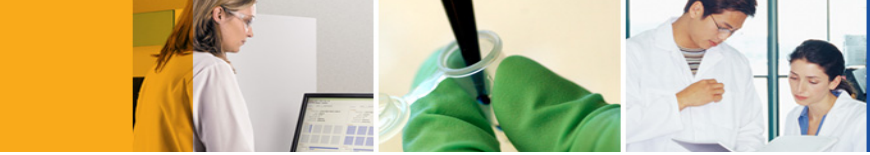
Dr. Victor Ambros
Professor
UMass Medical School



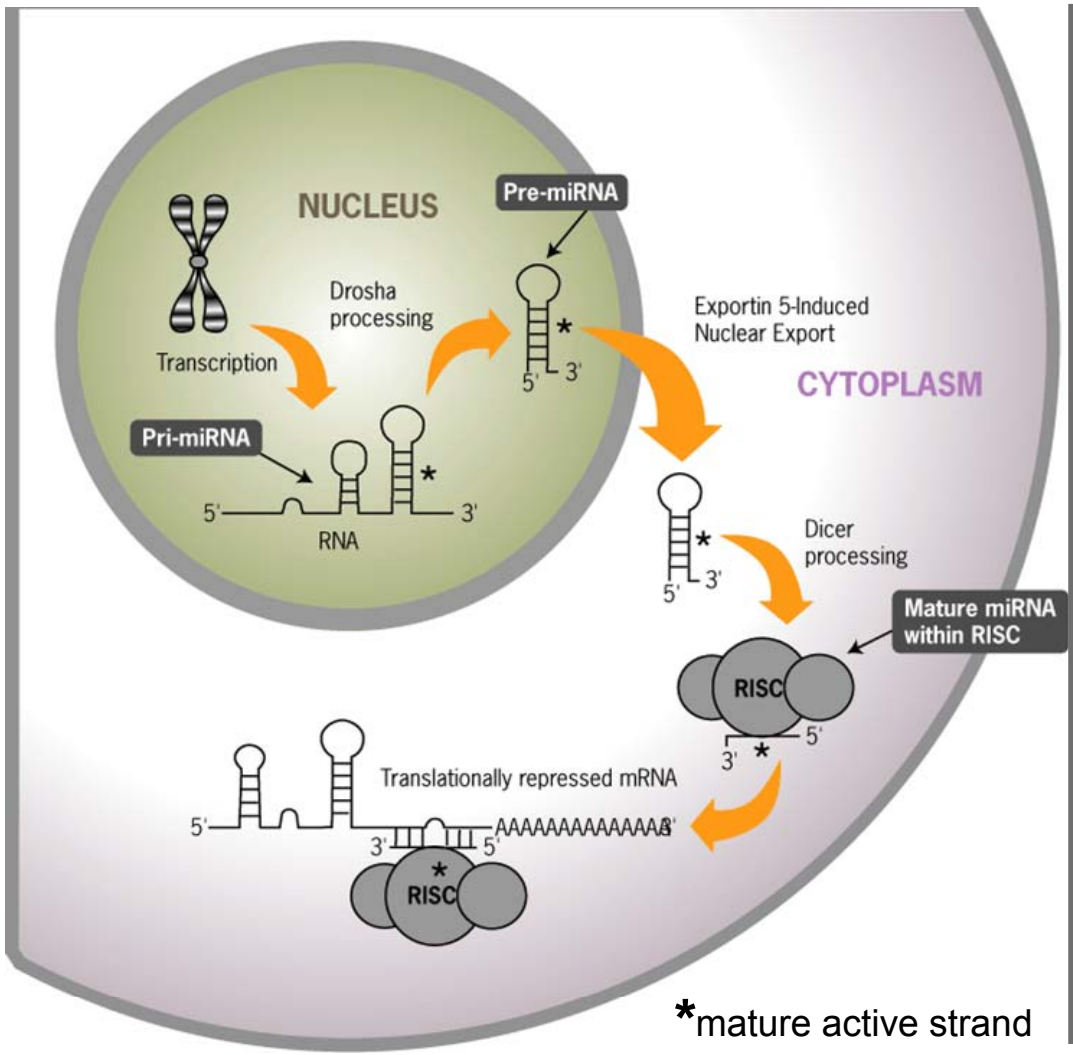
Lee et al. 1993; Wightman et al., 1993; Olson and Ambros, 1999

Definition of MicroRNAs

- ~ 21(18-25) nts in length
- Single-stranded
- Endogenous noncoding RNA molecules
- Processed from stem-loop precursors by RNase III enzymes Drosha and Dicer
- Highly conserved
- Target mRNAs largely for translational repression



Micro RNAs, Macro Significance



*mature active strand

Biogenesis:

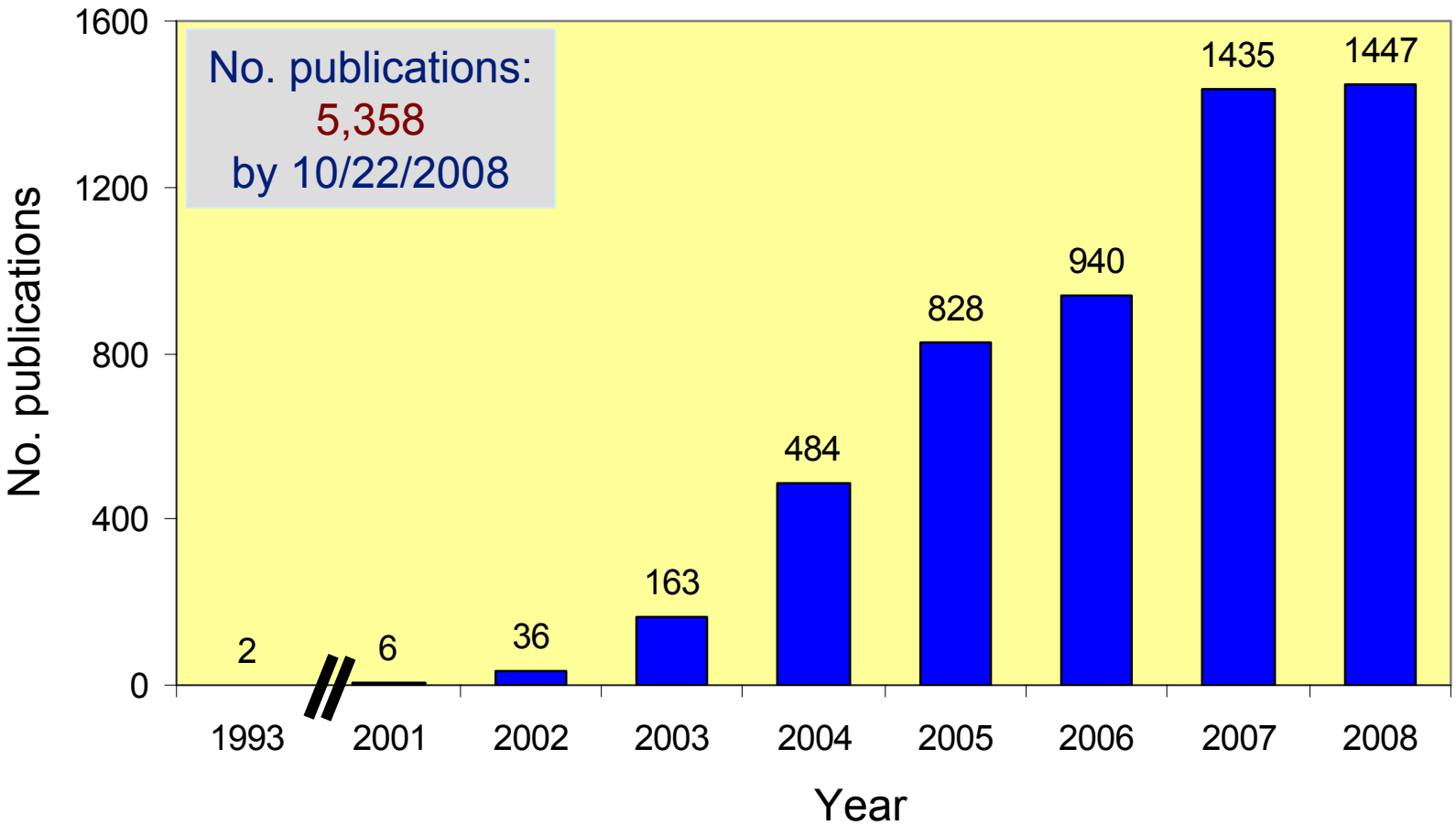
- Initial transcript, pri-miRs by Pol II from the genome
- Cleaved by Dicer into ~60 nt pre-miRs
- To ~22 nt mature miRNA by Drasha
- Post-transcriptional repression (or activation)

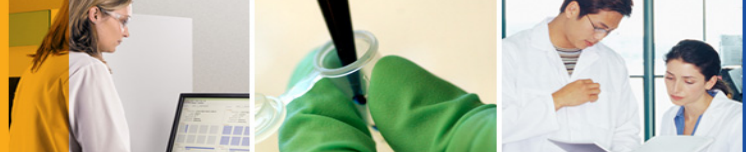
Functions:

- Development
  - Timing
  - Hematopoiesis
  - Neuron differentiation
  - Cell death and proliferation
- Diseases
  - Cancers
  - Diabetes
- Epigenetics (Chromatin modification)

What's new about miRNA research in 2007-8?

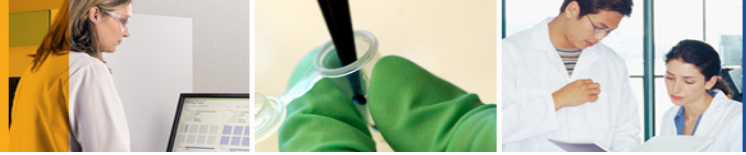
- MicroRNA publications increase very rapidly





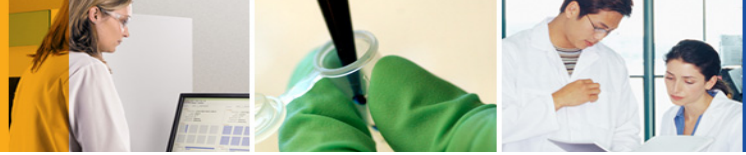
What's new about miRNA research in 2007-8?

- MicroRNA publications increased significantly
- **How many miRNAs are there in human?**
  - **By 2003**: **145** genes/ 152 mature miRNAs (Sanger Release 3.0)
  - **By 2004**: **192** genes/ 207 mature miRNAs (Sanger Release 5.1)
  - **By 2005**: **281** genes/ 319 mature miRNAs (Sanger Release 7.1)
  - **By 2006**: **411** genes/ 455 mature miRNAs (Sanger Release 9.0)
  - **By 2007**: **541** genes/ 733 mature miRNAs (Sanger Release 10.1)
  - **By 2008: 695** genes/ 866 mature miRNAs (Sanger Release 12.0)
  - **Total number of miRNA genes predicted:**
    - ~1,000 (Landgraf et al. 2007, Tuschl Lab)
    - >25,000 (Miranda et al. 2006, IBM Bioinformatics Group)



What's new about miRNA research in 2007-8?

- MicroRNA publications increased significantly
- How many miRNAs are there in human?
- **What regulates miRNAs** (He et al. 2007; Diederichs & Harber 2007; Ruby et al. 2007; Viswanathan et al. 2008)?
- **New role of miRNAs as tumor suppressors** (He et al. 2007; Mayr et al. 2007) and **tumor invasion** and **metastasis** (Ma et al. 2008)
- **MicroRNAs regulate stem cell self-renewal and differentiation** (Wang et al. 2007; Park et al. 2007; Yu et al. 2007; Yi et al. 2008; Tay et al. 2008)
- **MicroRNAs function in immune systems** (Li et al. 2007; Rodriguez et al. 2007; Thai et al. 2007)
- **Viral miRNAs regulate (HIV or HSV-1) replication** (Triboulet et al. 2007; Umbach et al. 2008)
- **MicroRNAs can target 3′ UTR, 5′ UTR and coding regions** (Lytle et al. 2007; Easow et al. 2007; Tay et al. 2008)



General approaches of microRNA research - 1

1. What miRNA genes to start with
   - 1.1 Select known miRNAs from Sanger's miRBase
   - 1.2 "Discover" novel miRNAs by *in silico* prediction
     - Assumptions:
       - Presence of hairpin structure
       - Phylogenetic conservation
       - Thermodynamic stability of hairpins
       - Genomic location, sequence and structure similarity to known miRNAs
     - Web servers: miRscan (Lim et al. 2003); ProMiR (Nam et al. 2005); BayesMiRNAfind (Yousef et al. 2006); Vir-Mir db (Li et al. 2008)
   - 1.3 Discover novel miRNAs by cloning and sequencing
     - RNA samples:
       - Enriched vs. total RNA; Normal (untreated) vs. patients (treated)
       - Caution: It is important to have relatively "pure" cell type
     - Methods:
       - Small RNA cloning followed by Sanger sequencing/NextGen sequencing
       - Follow-up validation using Northern, qPCR and microarray

General approaches of microRNA research – 2

2. How to identify miRNAs of interest

2.1 Differentially expressed miRNAs by using qPCR and microarray

- Normal vs. disease
- Treated vs. untreated
- Wild type vs. knockout (KO)

2.2 miRNAs that are predicted to target a mRNA at 3' UTR based on conserved seed sequence matches

- Some evidence suggested that miRNA could target 5' UTR or coding region (Lytle et al. 2007)

2.3 Direct IP of Ago/miRNA/mRNA complex (Karginov et al. 2007)

General approaches of microRNA research – 3

3. What are the targets of a miRNA

   3.1 Gain- or loss-of functions

   - miRNA mimics
   - miRNA inhibitors
   - Mouse KOs

   3.2 Luciferase reporter assays

   - With wild-type vs. mutant miRNA binding sites

   3.3 Expression analysis of punitive target(s) in the presence (enhanced) or absence (reduced) of miRNAs of interest

   - mRNA(s)
   - Protein(s)

   3.4 Phenotypic evaluation (proliferation etc.)

General approaches of microRNA research – 4

4. How a miRNA gene is regulated?

4.1 Discover a punitive miRNA regulator by using gain- or loss-function

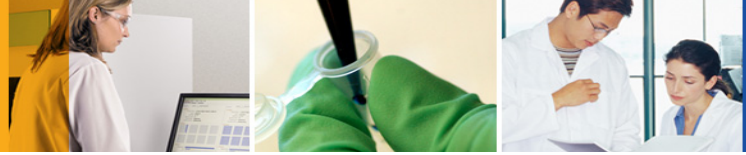
- Over-expression or reduced expression of a gene like transcriptional factor
- KOs of a gene

4.2 Identify or predict canonical binding sites in the punitive promoter regions of a miRNA gene

- *In silico* prediction
- ChIP seq or ChIP-on-chip

4.3 ChIP binding assays of miRNA promoters

4.4 Luciferase reporter assay for miRNA promoters



What's new about AB's miRNA tools?

Discovery

- Small RNA cloning kit
- Sequencing by **SOLiD**

Sample preparation

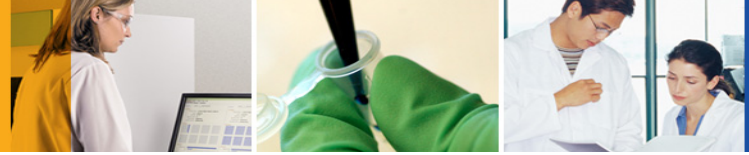
- **MicroRNA Cells-to-Ct** kit (4391848)
- **RecoverAll** for FFPE (AM1975)
- **mirVana**
  - P/N: AM1560
  - Paris (P/N: AM1556)
- **Synthetic miRNA ref panel** (4388891)

Expression analysis

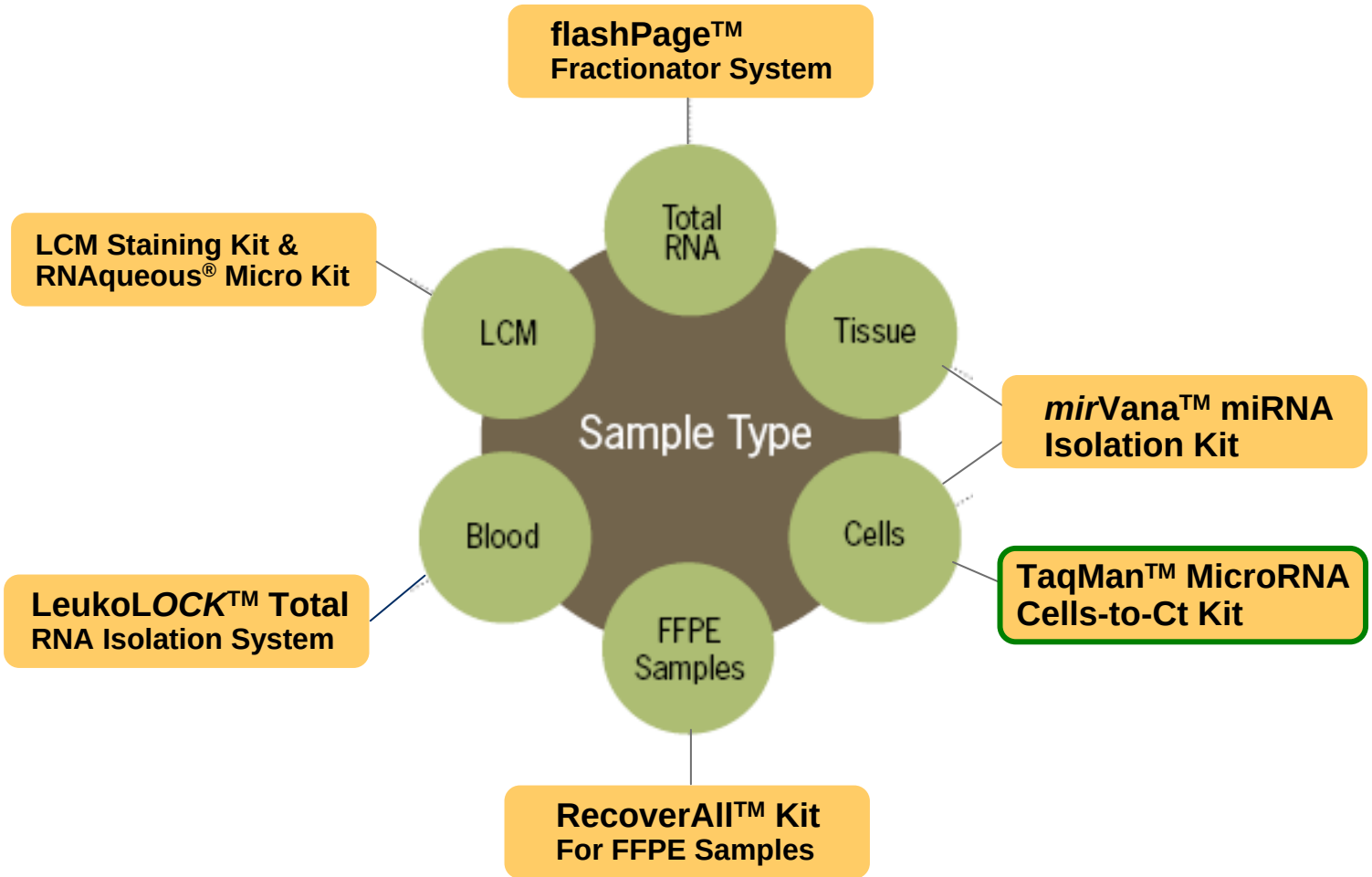
- **Individual TaqMan miRNA assays v10**
- **TaqMan MicroRNA Arrays**
  - Human
  - Mouse/rat
- **Megaplex PreAmp MicroRNA Assays** up to single cells
- **Custom TaqMan sRNA assays**
  - Novel miRNAs
  - siRNAs etc.

Functional analysis

- **Anti-miR™** v3 (4385914)
- **Pre-miR™** v3 (4385830)
- **pMIR-REPORT™** miRNA Expression Reporter System (AM1975)
- **Protein analysis**
  - PLA
  - Western SuperStar™ Immunodetection



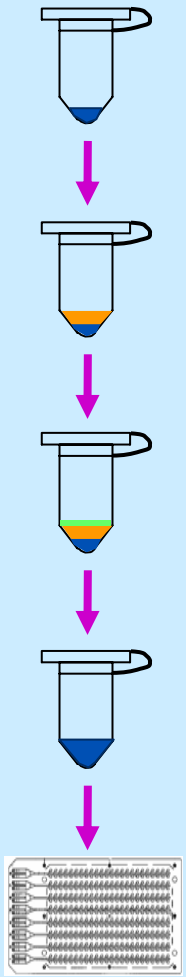
AB's Total Solutions For MicroRNA Sample Prep



MicroRNA Cells-to-Ct Kit (P/N: 4391848)

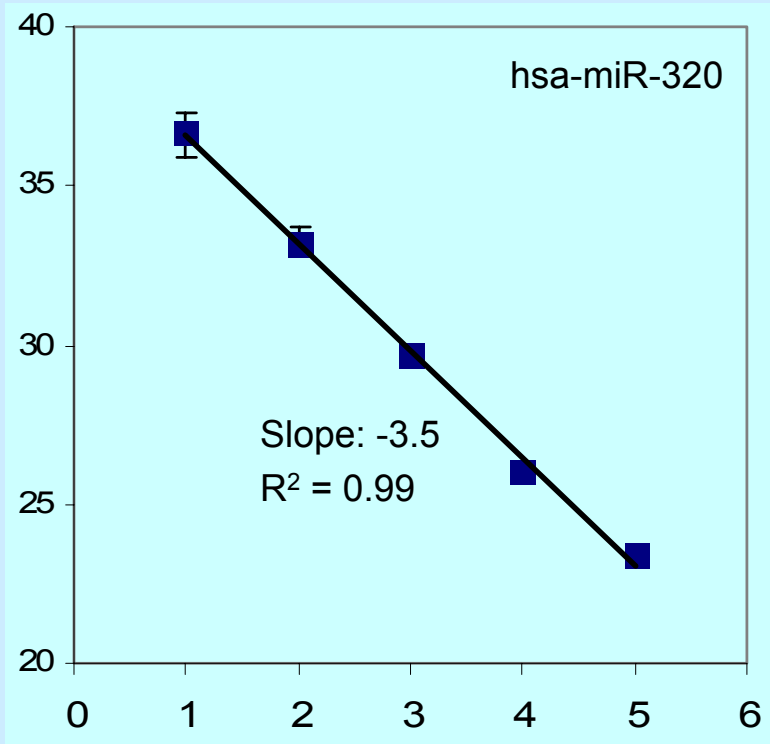
Workflow

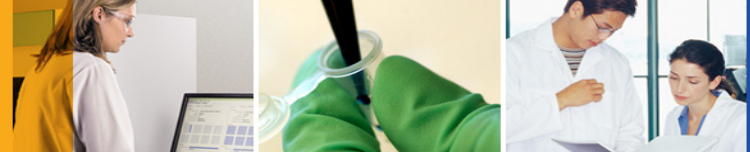
1. Pellet and wash cells with 1X PBS
2. Add 50 uL lysis solution, mix, and incubate 8 min
3. Add 5 uL Stop solution, mix, and incubate 2 min
4. Perform multiplex RT by adding up to 40% lysate
5. Perform TaqMan PCR on Arrays

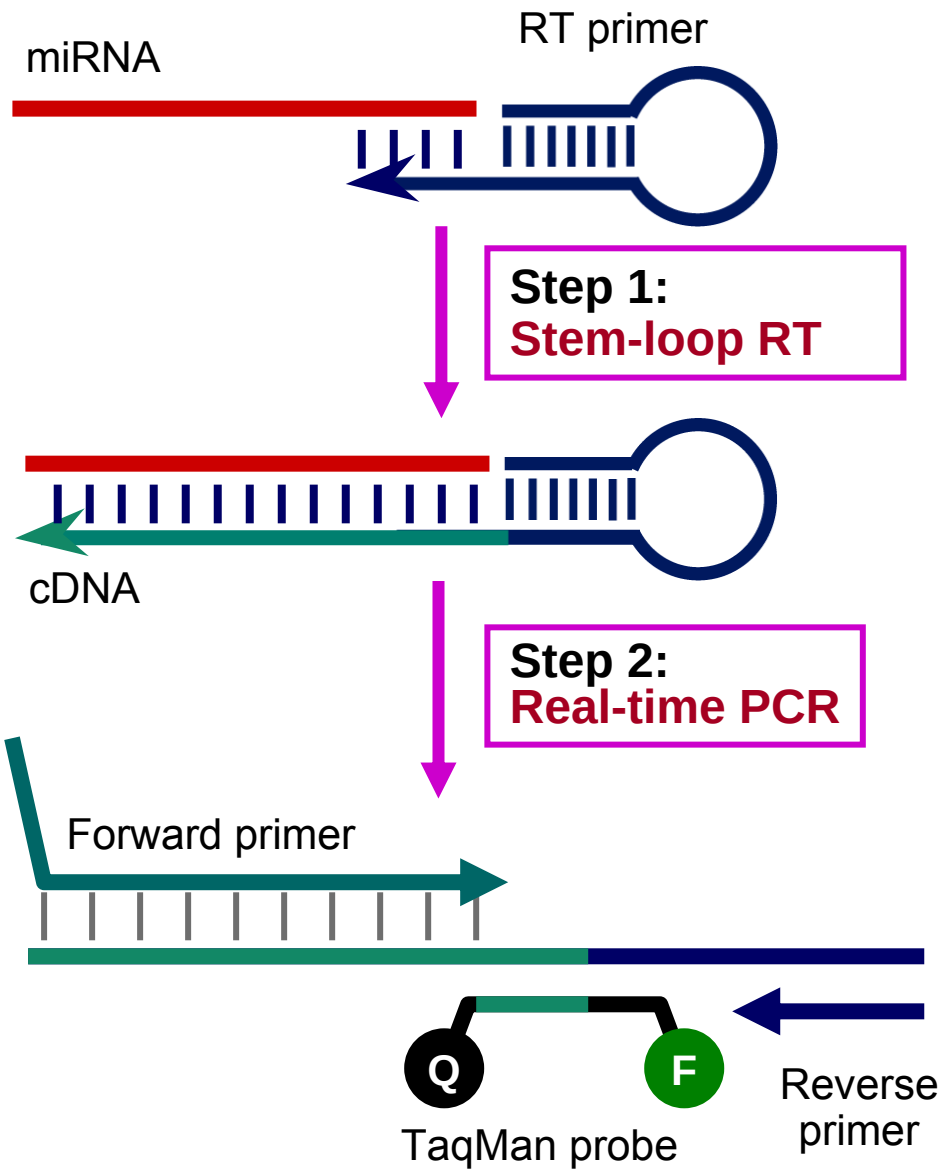


Performance

Linear quantitation of hsa-miR-320 over a cell input range of 10 - 10^5 HeLa cells per lysis solution





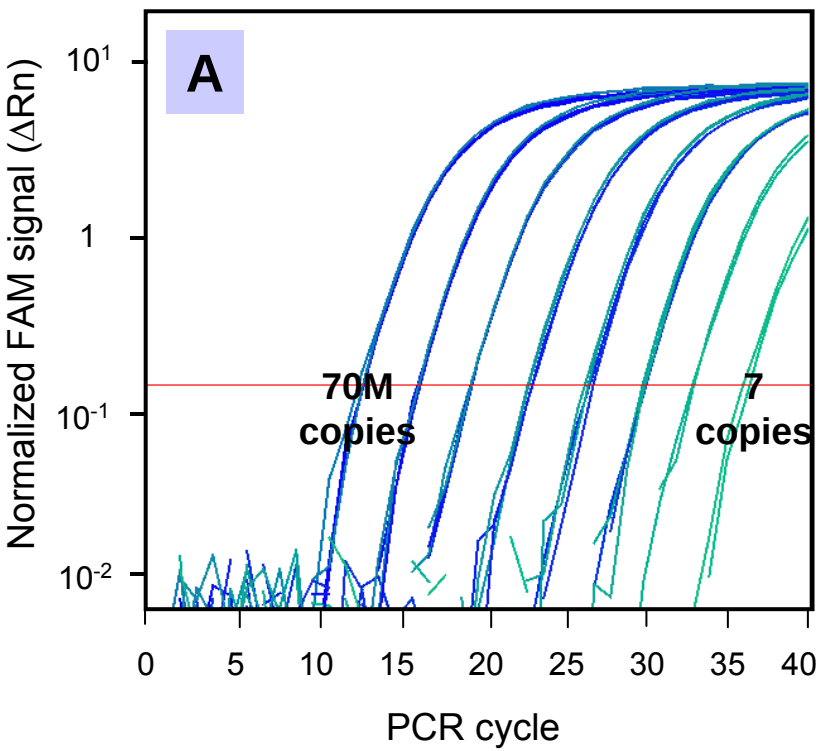


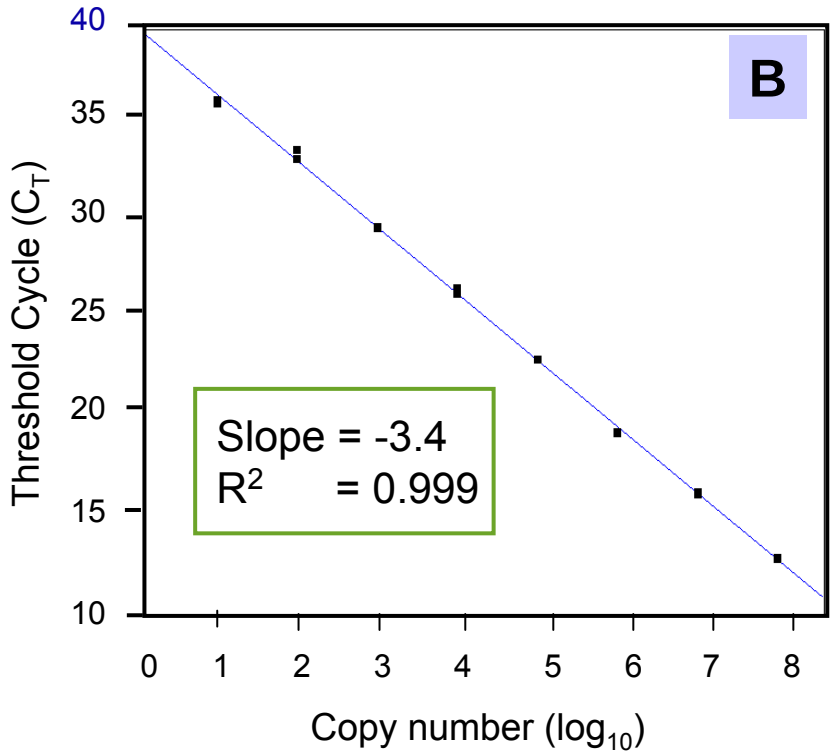
Singleplex TaqMan MicroRNA Assays

Chen et al. (2005) Nucleic Acid Res. v33: e179

MicroRNA quantitation – Up to 7-log dynamic range

Synthetic lin-4 miRNA input from 7 to 70M copies in PCR



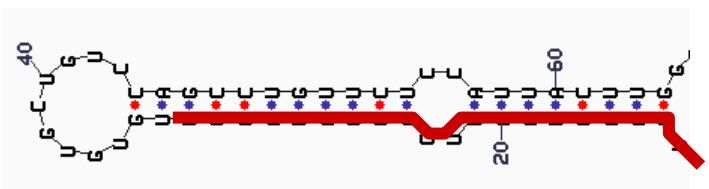


Assays are specific for mature miRNAs

ID	Synthetic RNA (No. copies)		TaqMan miRNA Assay (C_T)
	Mature miRNA	Precursor	
let-7a	1.5×10^8	0	**16.5**
	0	1.5×10^8	**29.5**
	0	0	Not detectable

Mature miRNA: **UGAGGUAGUAGGUUGUAUAGUU** 22 nts

MicroRNA precursor:

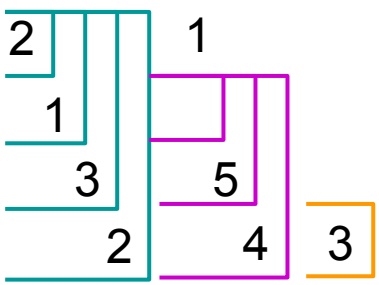


55 nts

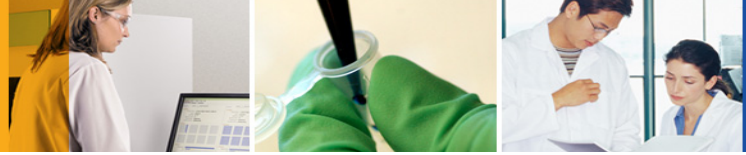
Single base discrimination

		Synthetic miRNA template					
		let-7a	let-7b	let-7c	let-7d	let-7e	
miRNA assay	let-7a	100.0	0.3	3.7	0.0	0.0	Relative detection (%)
	let-7b	0.0	100.0	0.3	0.0	0.0	
	let-7c	0.0	2.5	100.0	0.1	0.0	
	let-7d	0.1	0.0	0.0	100.0	0.0	
	let-7e	0.0	0.0	0.0	0.0	100.0	

let-7a ugagguaguagguuguauaguu
let-7b ugagguaguagguugugugguu
let-7c ugagguaguagguuguaugguu
let-7d agagguaguagguugcauagu
let-7e ugagguaggagguuguauagu



Relative detection (%) was calculated based on C_T difference between perfectly matched and mismatched assays

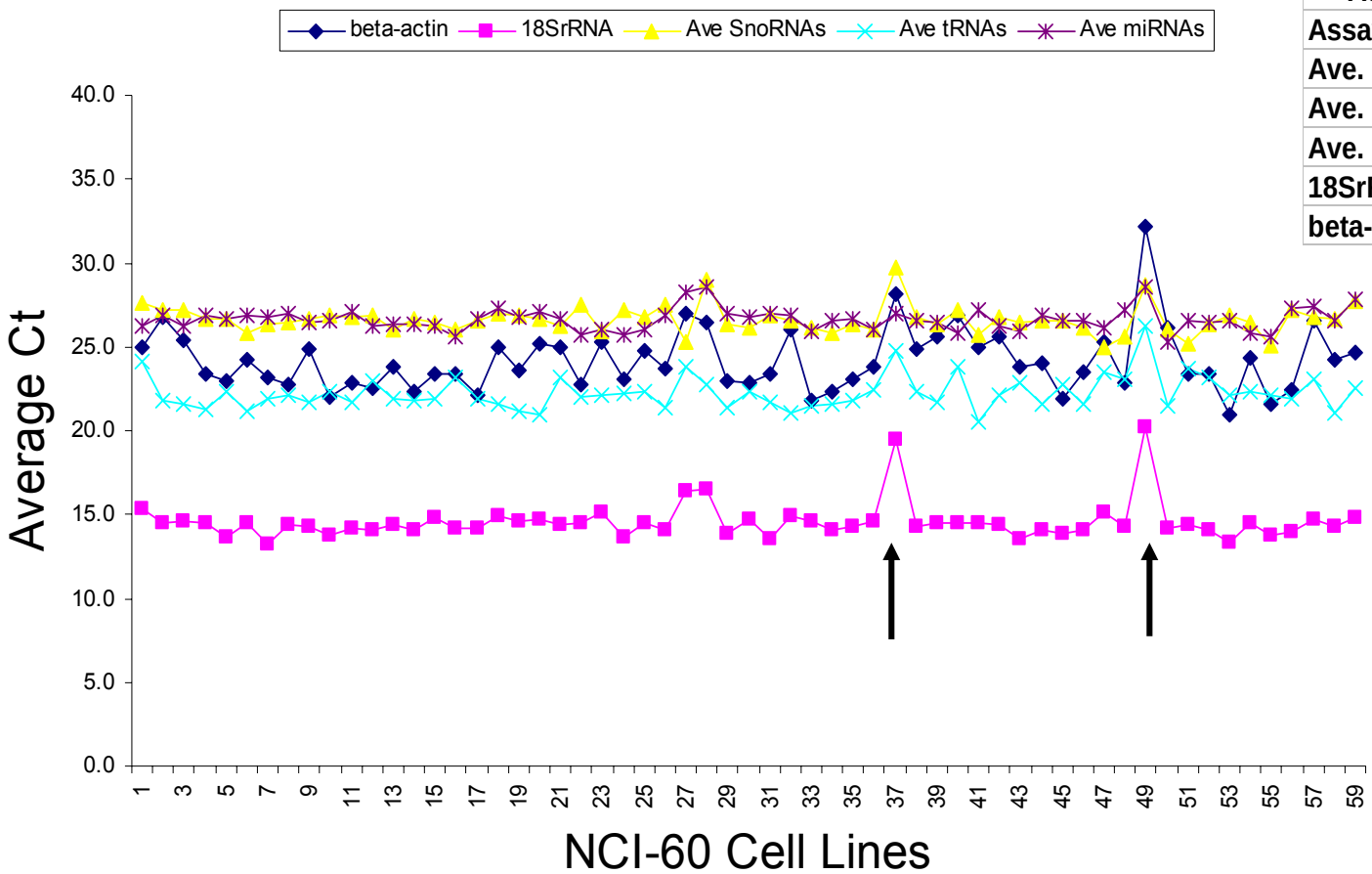


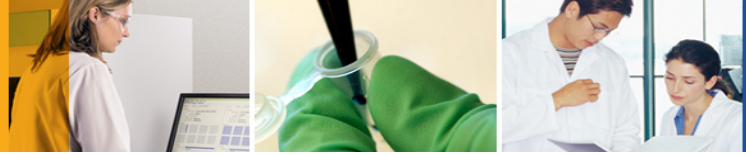
Recommendations for analyzing miRNA expression data

- Endogenous control genes recommended
  - snoRNA assays: 18, 10, and 5 for human, mouse and rat, resp.
    - Human: U6, RNU48, RNU44, U47, and RNU6B
    - Mouse: snoRNA-202, snoRNA-234, snoRNA-420
  - Least variable miRNA genes
    - Human/mouse tissues: miR-152, -186, -25, -92, -26b, -16
    - Human/mouse cell lines: miR-374, -16, -93, -186, -26b, -92
  - 18S rRNA or U6
- Normalize your C_T values using endogenous controls
- Use ΔC_T (miRNA C_T – Endo C_T) or fold-change ($2^{\Delta CT}$) for clustering or estimating copy No. changes in samples

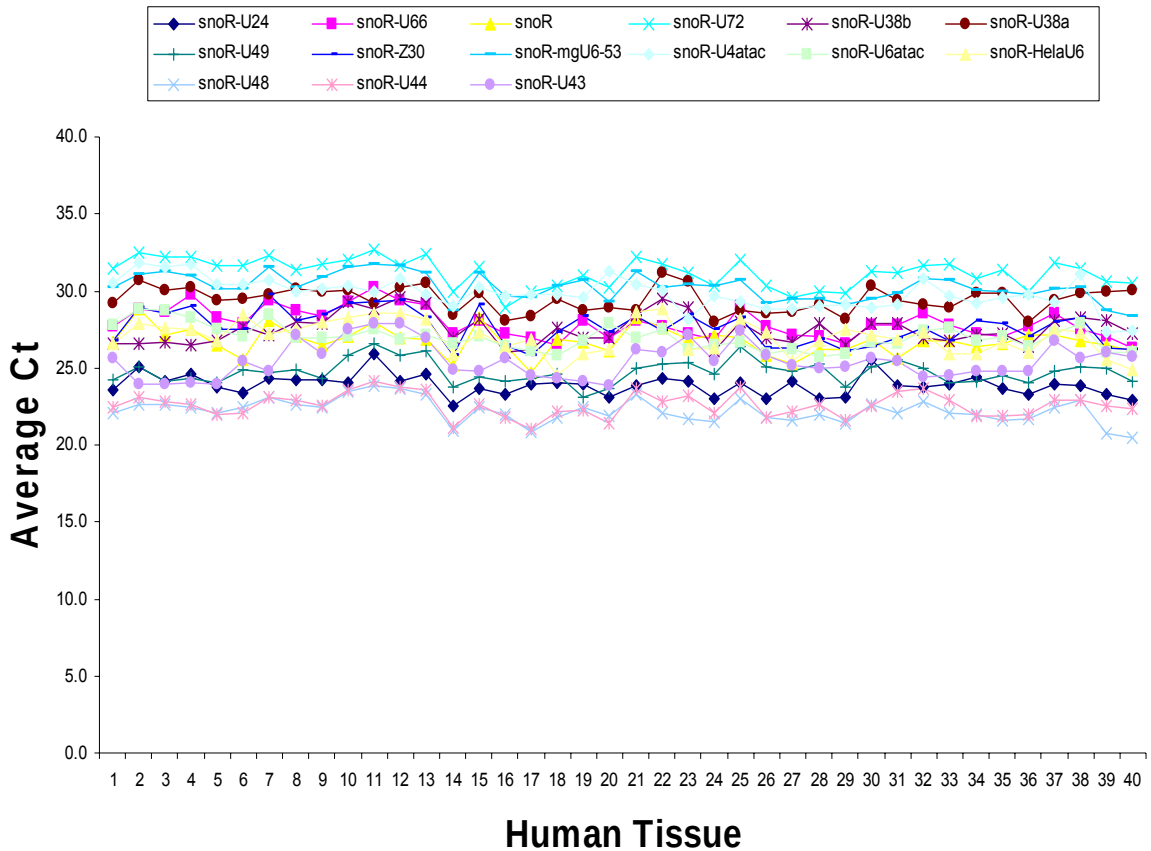
Expression pattern comparison for different types of endogenous controls in NCI-60 cell lines

Across 59 Cell Lines		
Assay Type	Ave.	StDev
Ave. miRNAs	26.6	0.7
Ave. snoRNAs	26.6	0.8
Ave. tRNAs	22.3	1.0
18SrRNA	14.6	1.2
beta-actin	24.2	1.9



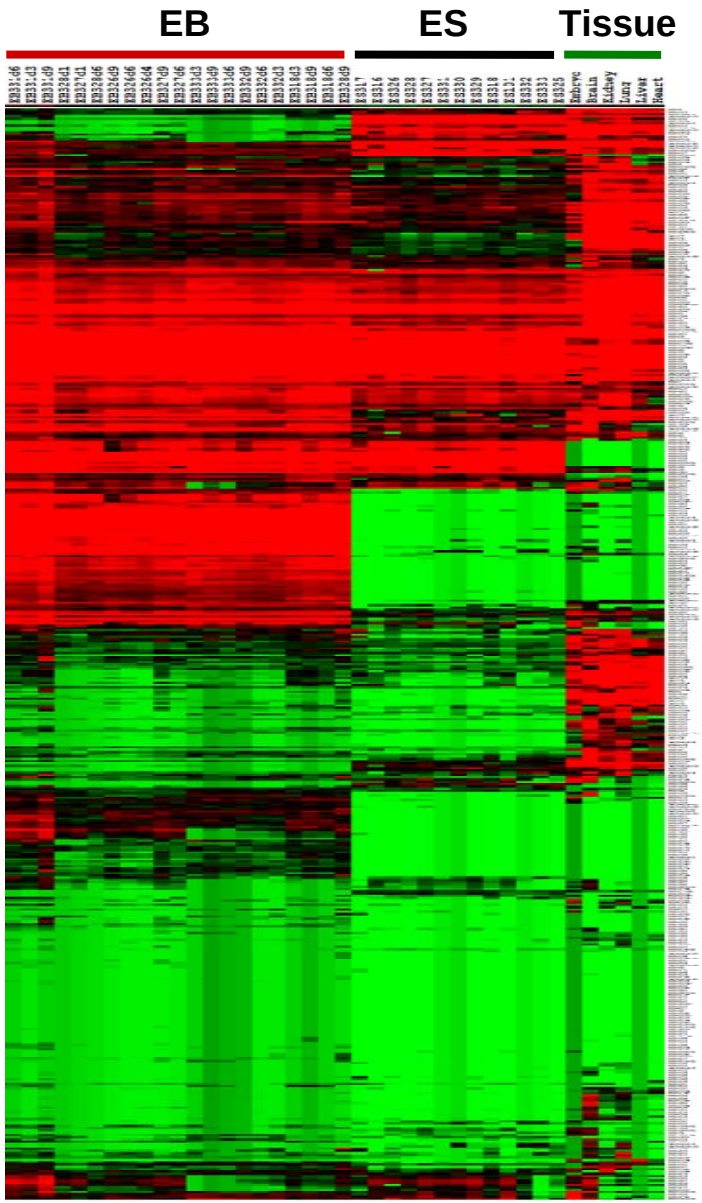


snoRNA Expression in 40 Human Tissues



Across 40 Tissues		
Assay Type	**Ave.**	**StDev**
snoR-U24	23.9	0.7
snoR-U49	24.7	0.7
snoR-U44	22.6	0.8
snoR-U48	22.2	0.8
snoR-U6atac	27.0	0.8
snoR-U38a	29.4	0.8
snoR	26.7	0.9
snoR-mgU6-53	30.3	0.9
snoR-U72	31.2	0.9
snoR-U66	27.9	1.0
snoR-U38b	27.5	1.0
snoR-U4atac	29.8	1.0
snoR-HelaU6	27.0	1.1
snoR-Z30	27.6	1.1
snoR-U43	25.5	1.1

MicroRNA profiles classify mouse ES and differentiated cells



5. ES differentiation-related miRNAs

1. Highly expressed miRNAs

4. Stem cell specific miRNAs

5. ES differentiation-related miRNAs

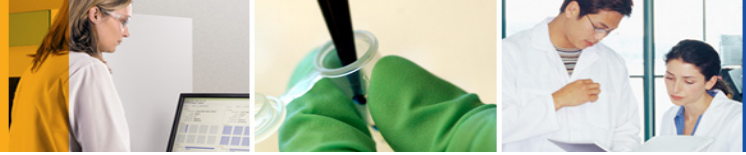
3. Somatic Tissue specific miRNAs

5. ES differentiation-related miRNAs

2. Rarely expressed miRNAs

3. Somatic Tissue specific miRNAs

Mammalian Genome (2007) 18:316-327

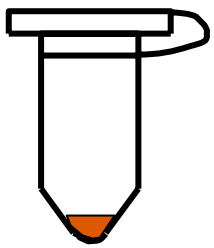


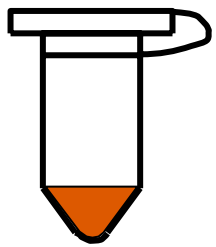
MegaPlex PreAmp TaqMan MicroRNA Assays For Single Cell Gene Expression Profiling

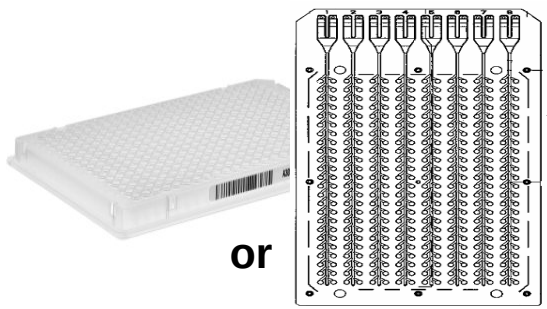
Step 1:
384-plex
RT

Step 2:
384-plex
PreAmp

Step 3:
Singleplex
TaqMan







or

RT
(10 copies/cell)
→ 16 cycles, ~64K-fold →
PreAmp
($6x10^5$ copies)
→ $6x10^5/384/30$ →
PCR
(50 copies/well)

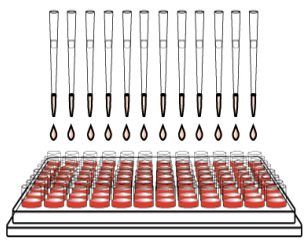
RT
(10 copies/cell)
→ 10/384 →
PCR
(0.0 copies/well)

Reproducibility of Single Cell Expression Profiling Method

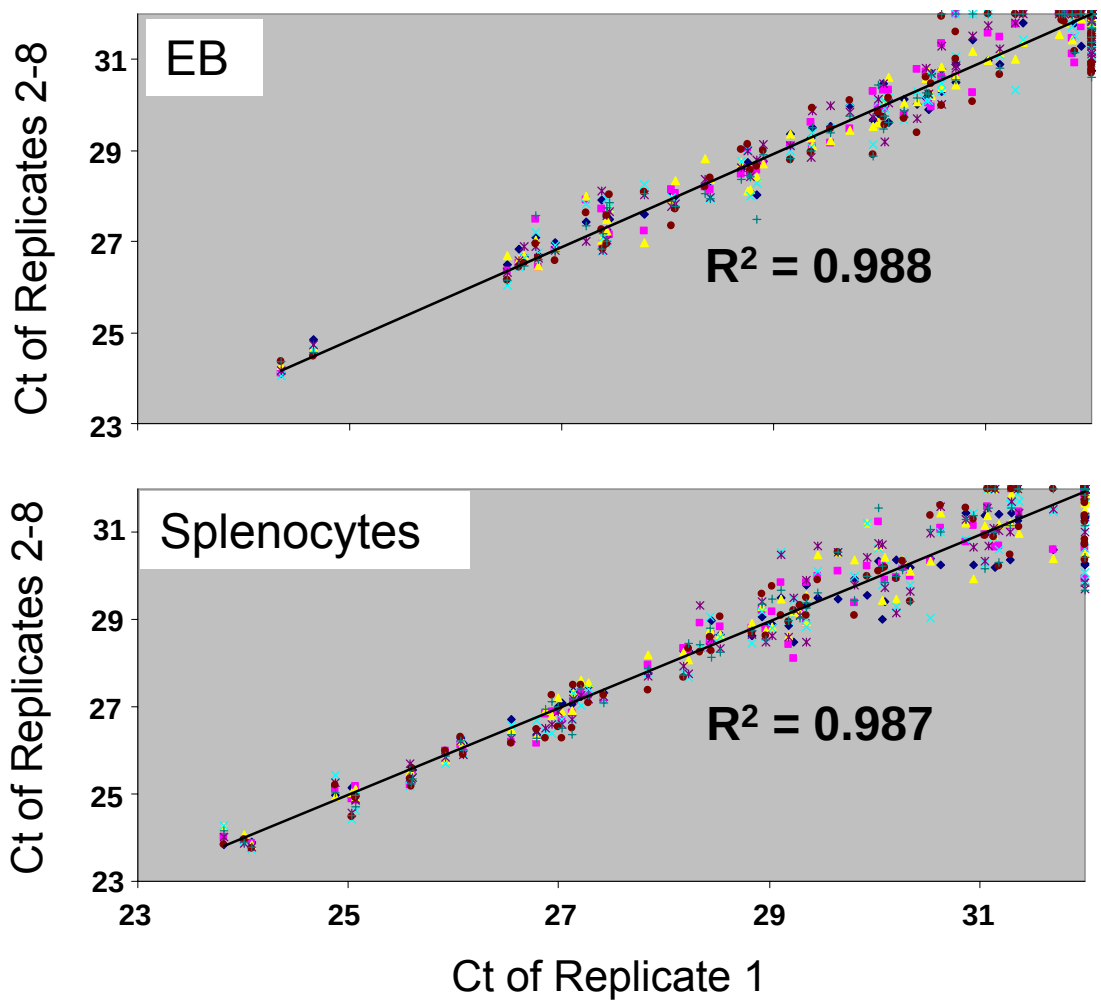
Dilute total RNA to single cell level (40 pg/well)

Repeat pipeting **8 times**

Mimic single cell/well



Expression Profiling

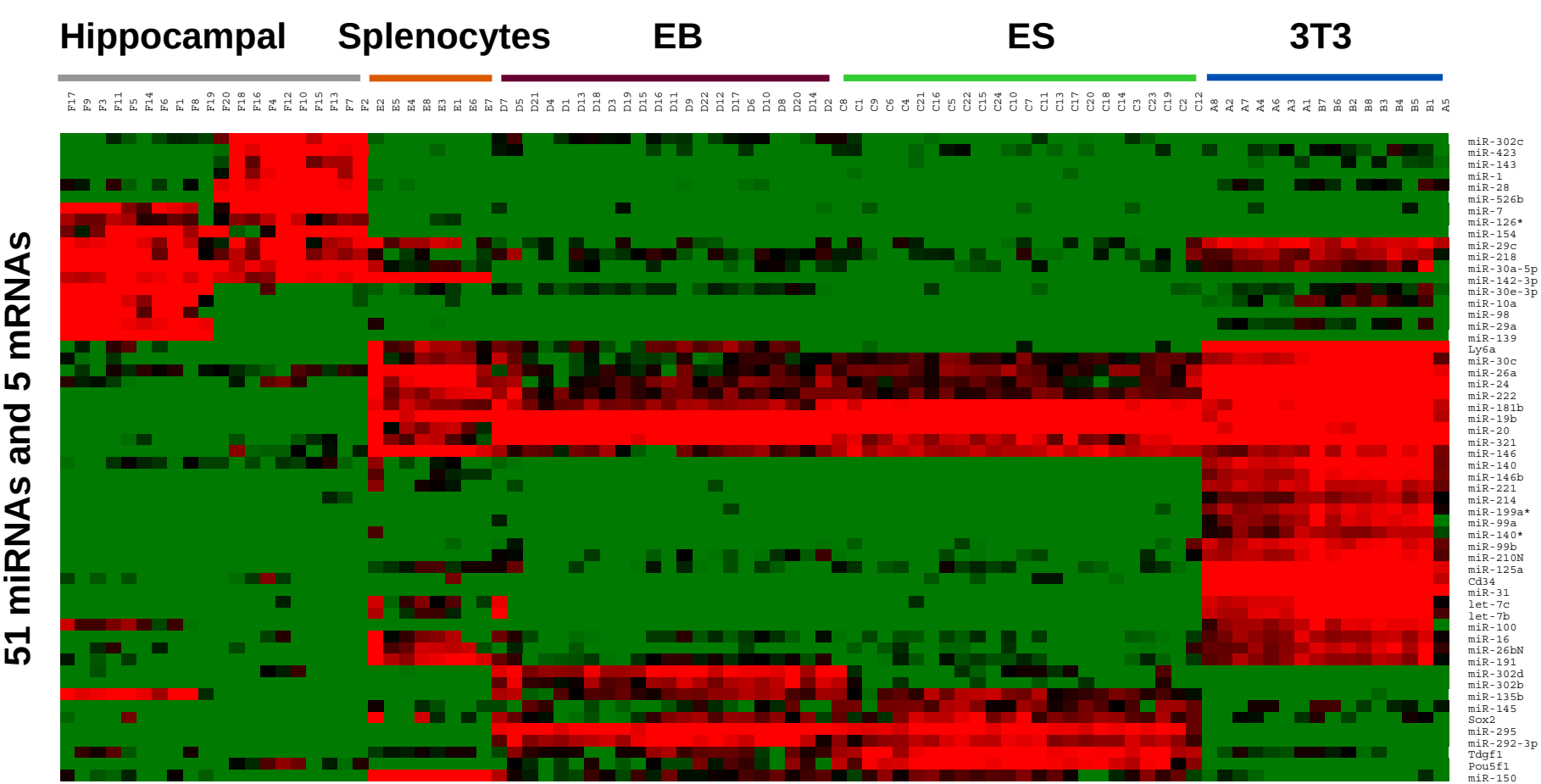


Questions?

- Is miRNA expression variable from ES cell to ES cell?
- Can miRNAs be used as biomarkers to distinguish:
  - ES vs differentiated cells?
  - Stem cells vs. cancer stem cells?

Embryonic stem (ES) cells

Single cell miRNA & mRNA expression profile in 90 individual ES, EB, and somatic cells

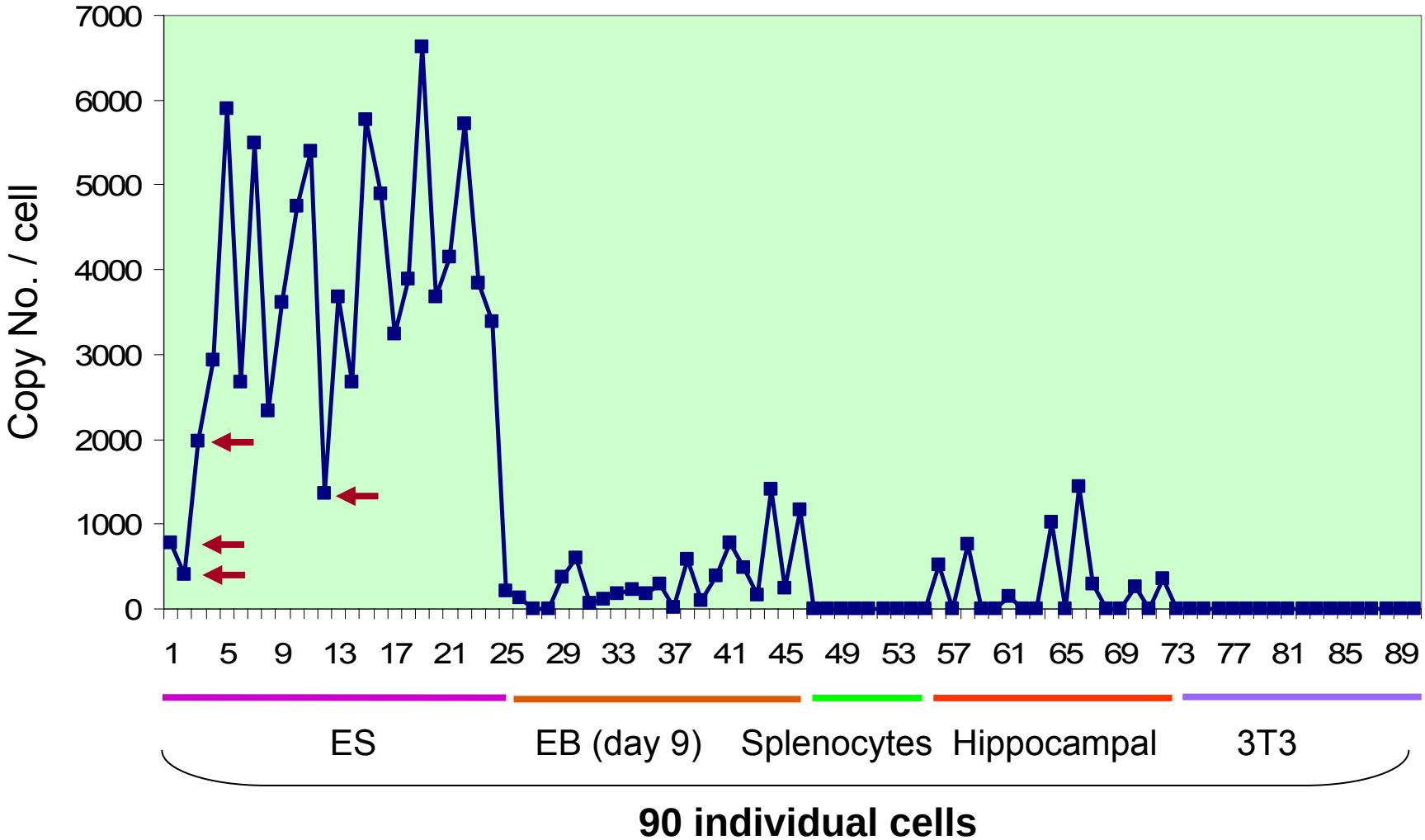


Single cell miRNA expression in ES, EB, and somatic cells

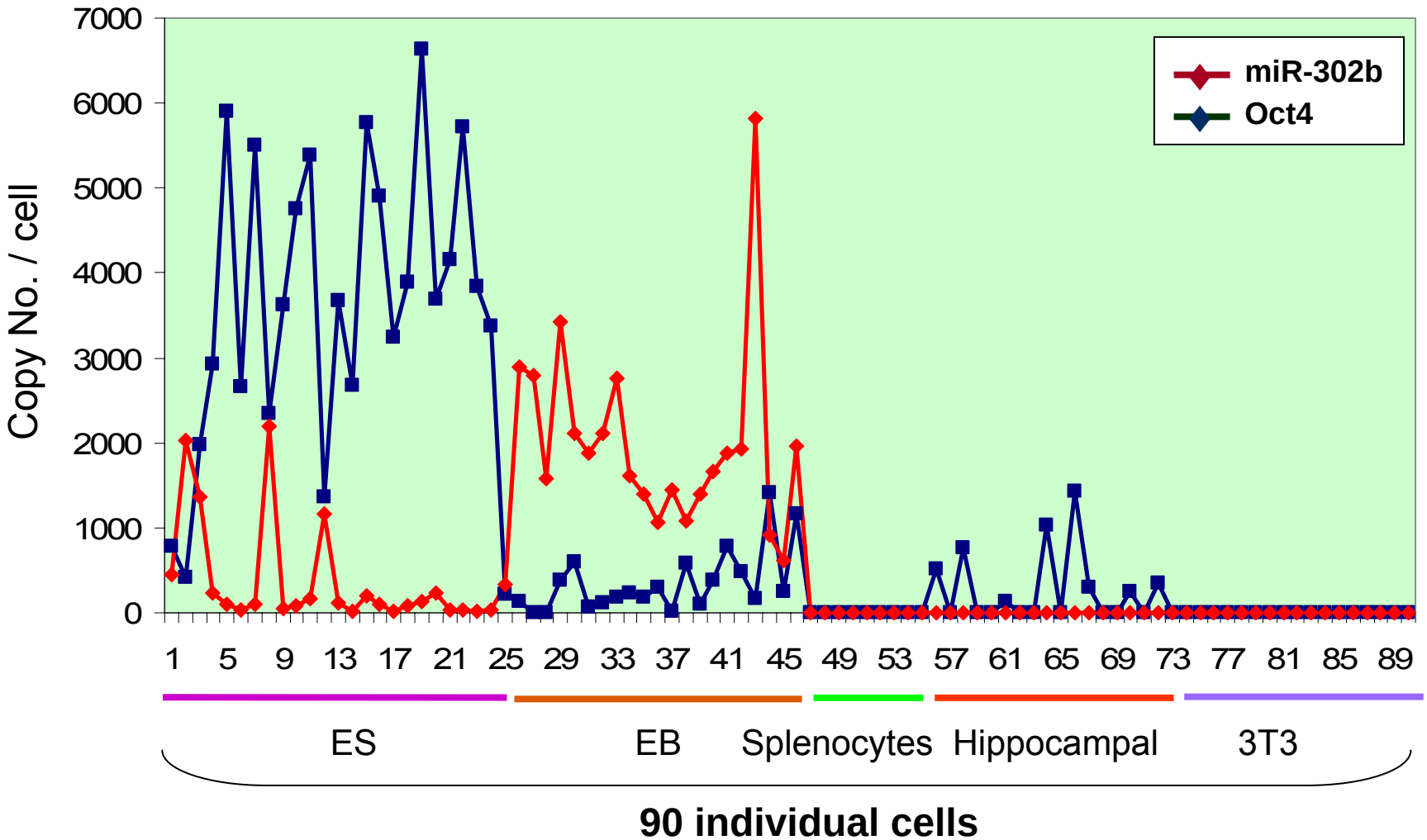
		ES	EB	3T3	Splenocytes	Hippocampal cells
No. cells analyzed		24	22	8	8	20
Ave. copy No./cell		210	300	1270	960	900
% of miRNAs with	0 copy/cell	48	49	49	51	45
	>0, ≤10 copies/cell	15	11	9	8	8
	>10, ≤100 copies/cell	19	`20	13	21	16
	>100, ≤1,000 copies/cell	14	16	15	12	20
	>1,000 copies/cell	5	4	14	8	11
Inter-cell CV (%)		18	25	17	24	46

Note: A Ct cutoff of 32 was used to calculate inter-cell CV (%) of miRNA expression levels.

Expression pattern of ES marker gene Oct4 in single ES, EB, and somatic cells



Negative correlation of Oct4 and miR-302b expression



Summary

- TaqMan single cell miRNA assays are quantitative & reproducible
- MicroRNA expression signatures can classify ES and differentiated cells. Expression level of miR-302b and OCT4 may reflect ES cell differentiation status
- The level of miRNA expression varies in individual ES cells, suggesting heterogeneities of ES cell population
- Simultaneous quantification of mRNAs and miRNAs from cell lysate demonstrated successfully
- Finally, results suggest that microRNAs may be better ES biomarkers

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Assays Project Teams

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Notes: