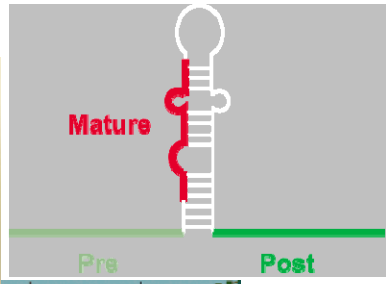
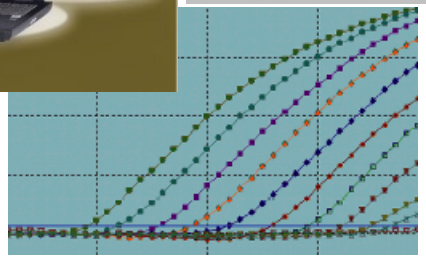
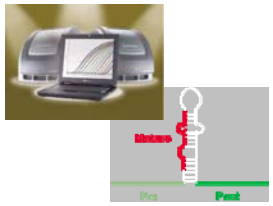
Microarray and QPCR applications for miRNAs





Cathy Cutler
Field Application Scientist

Stratagene Products Division

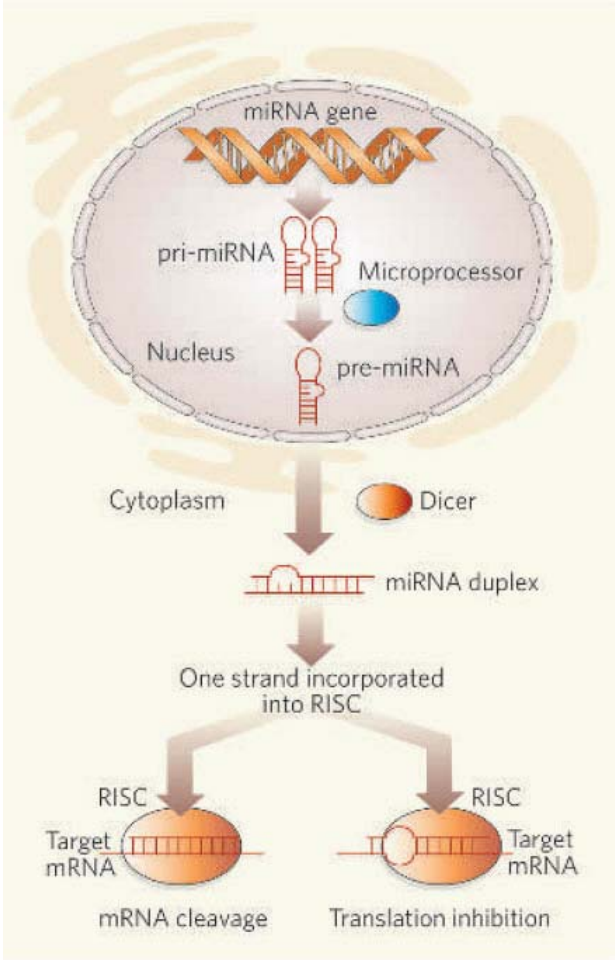


Introduction to miRNA

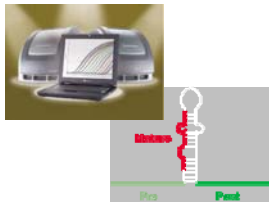
What are microRNAs

- Non-coding small RNAs (19-30 nt)
- 3 different forms:
  Pri- and pre-miRNA: hairpin shaped
  mature miRNA: bound to RISC complex
- Act in repression of translation or direct mRNA degradation
- Implicated heavily in disease (eg. cancer), development, apoptosis, proliferation and differentiation
- Involved in viral infections
- More than 700 human miRNAs identified (Sanger miRBase 10.0)
- Implicated in regulation of up to 30% of all human genes
- Projected $100M to be awarded by the NIH for miRNA-related research in 2008*

*Source: NIH CRISP database at http://crisp.cit.nih.gov/



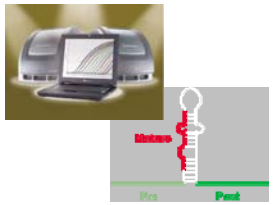
Cancer Genomics: Small RNAs with big impacts from Nature 435: 745-746 (9 June 2005)



Introduction to miRNA

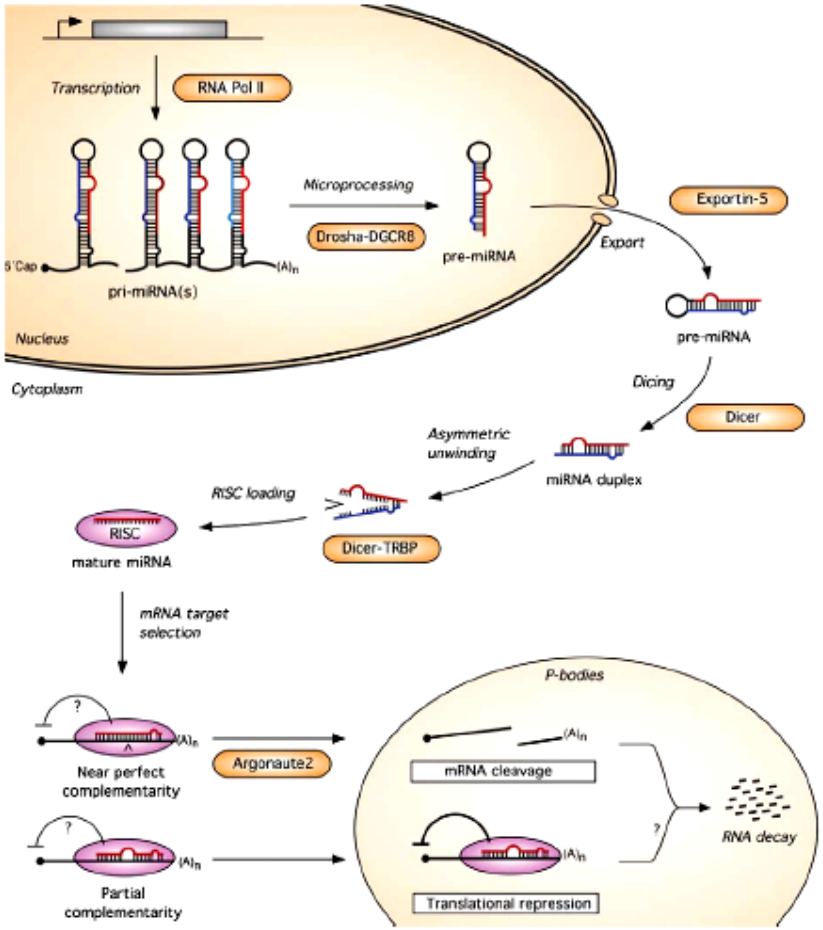
Why Are miRNAs Important?

- Predicted to regulate expression of 30% of human genes
- Act as key regulators of many cellular processes, such as:
  - **Early development**
  - **Cell proliferation and apoptosis**
  - **Fat metabolism**
  - **Cell differentiation**
- Involved in viral infection processes
- Differentially expressed miRNAs are reported in many human cancers
- > 50% of miRNA genes are located within regions associated with amplification, deletion, and translocation in cancer (Calin, G.A. et al PNAS 2004 101:2999-3004)

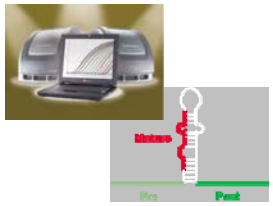


Introduction to miRNA
Biogenesis in mammals

E. Wienholds, R.H.A. Plasterk / FEBS Letters 579 (2005) 5911–5922



- Expression may be regulated by transcription factors
- monocistronic and polycistronic (40%) miRNA genes expressed by pol II promoter
- Intronic miRNA genes (~50%) regulated with mRNA that flanks miRNA
- miRNA transcripts can contain more than one miRNA



Introduction to miRNA

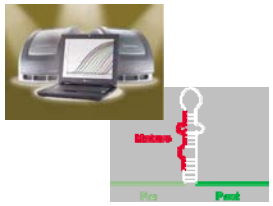
Mode of Action – Repression of Translation

miRNA

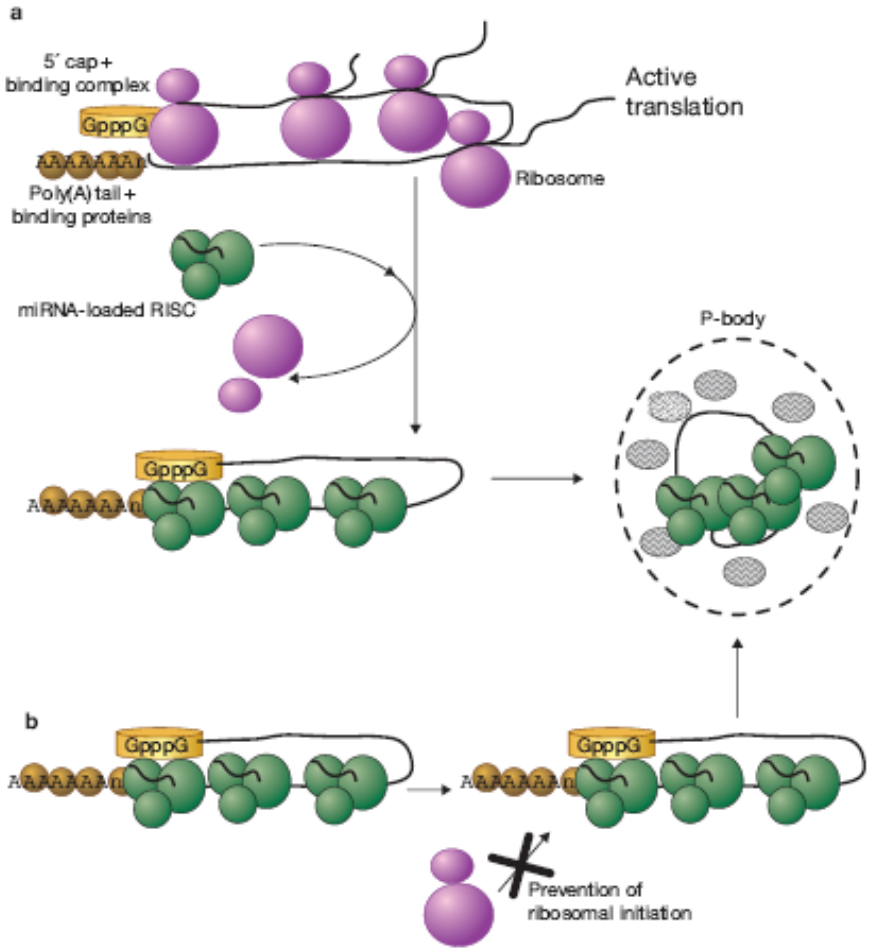
miRNA bound by RISC complex

RISC bound miRNA binds target mRNA

Bound miRNA-RISC blocks translation of mRNA by ribosome



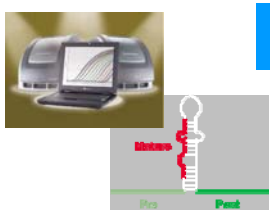
Introduction to miRNA
Mode of Action – RNA Degradation



P-body
contains RNA that can no longer be translated and enzymes that are required for RNA decay and degradation

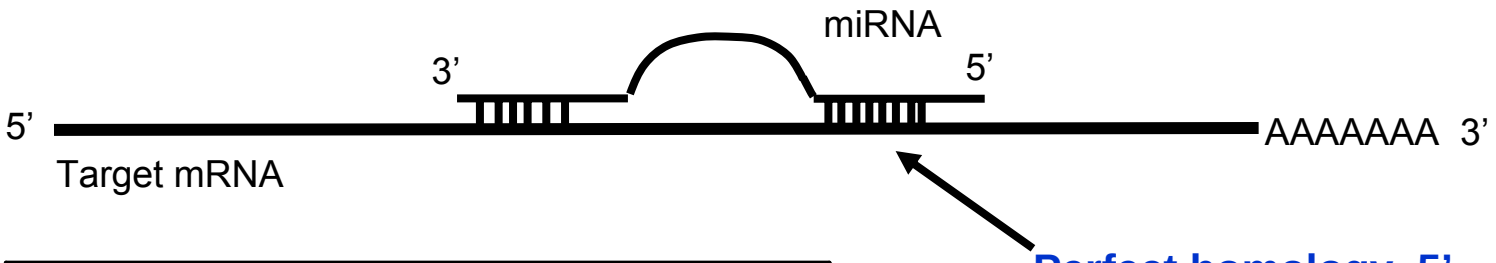
mRNA degradation allows for detecting effects of miRNA using microarrays

Rossi, J J 2005 Nature Cell Biol 7(7):643-644



Interaction of miRNA with mRNA

Family Grouping by Seed Region

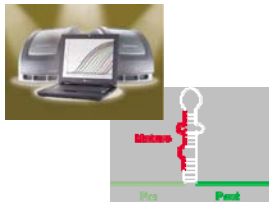


miRNA	nucleotide sequence (5’-3’) seed
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU
hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU
hsa-let-7d	AGAGGUAGUAGGUUGCAUAGU
hsa-let-7e	UGAGGUAGGAGGUUGUAUAGU
hsa-let-7f	UGAGGUAGUAGAUUGUAUAGUU
hsa-miR-98	UGAGGUAGUAAGUUGUAUUGUU
hsa-let-7g	UGAGGUAGUAGUUUGUACAGU
hsa-let-7i	UGAGGUAGUAGUUUGUGCUGU

Perfect homology, 5’

6-8nt of miRNA (seed)

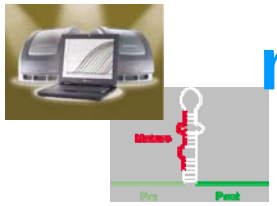
- **one miRNA may regulate hundreds of genes**
- **a gene may be regulated by more than one miRNA**



Introduction to miRNA

miRNA Registry

- http://www.sanger.ac.uk
- Searchable database of all published miRNA sequences and annotation
- Mouse and human are highly conserved
- Human is not conserved with plants
- Data can be downloaded from ftp site:
  - ftp://ftpsanger.ac.uk/pub/mirbase



miRNA qRT-PCR Methods and Microarray Methods

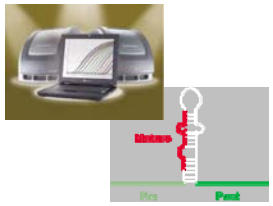
- QRT-PCR miRNA detection methods are more specific than microarray methods.

- Microarray methods tend to show higher cross-hybridization which can lead to false positives. Data should be confirmed by QRT-PCR

- Microarray methods are ideal for profiling a small number of samples for many miRNAs

- QRT-PCR methods are ideal for:

Profiling a large number of samples against a small number of miRNAs
Profiling a moderate number of samples against a moderate number of miRNAs

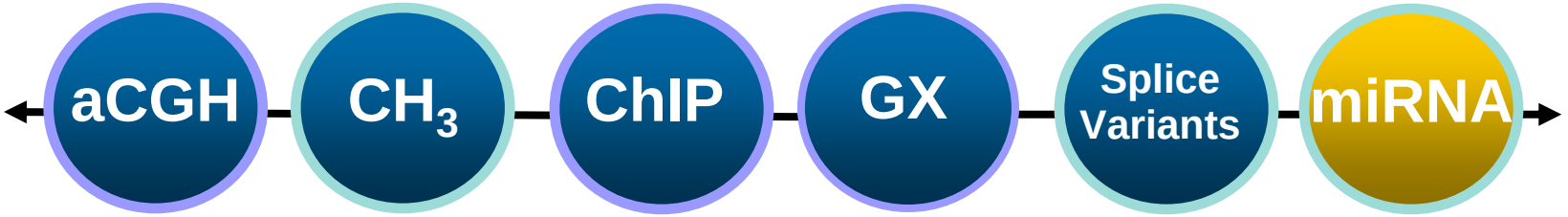


Choose from a comprehensive set of microarray applications

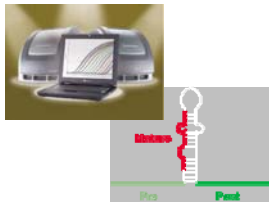
From one-dimensional to multi-dimensional …

with multiple applications …

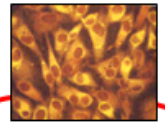
DNA RNA



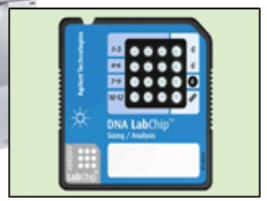
Copy number	Methylation	Transcription Factors	mRNA	mRNA isoforms	miRNA Profiling
• Conduct high-resolution, genome-wide profiling of DNA copy number changes	▪ Discover and monitor epigenetic modifications	• Elucidate the role that protein-DNA interactions play in transcription, replication, modification and repair	• Explore gene transcription on a genome-wide basis	• Perform global interrogations of the transcriptome and identify alternative splice forms	• Study microRNAs and the role they play in gene regulation



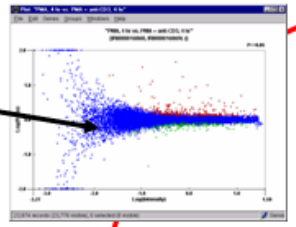
Microarray work flow



Bioanalyzer:
Measure picograms RNA

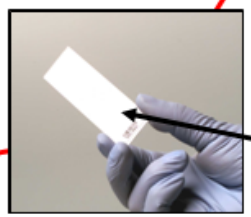


Resover software
Gene Spring



Labeling Kits:
50ng total RNA
2ng total RNA

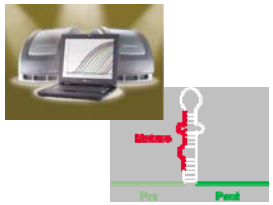
Microarray scanner:
3-5X sensitivity
High throughput



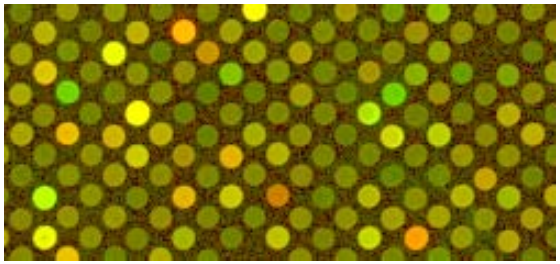
Hyb chambers:
Hybridization uniformity

Oligo microarrays:
60mer sensitivity
Content flexibility
<1copy/cell x 10^6 cells

Data Workflow



Grid Template
Grid File
FE Protocol



16-bit Tiff Image
(uncompressed)

Agilent and non-Agilent microarrays scanned on Agilent Scanner

Agilent microarrays scanned on GenePix Scanner

Grid and Measure Spots
Reject Outlier Pixels
Subtract Background
Correct Dye Biases

Feature Extraction Software

QC Report
Grid
Shape
Text
JPEG
MAGE-ML
GEML

Feature Extraction Result Files

CGH analytics

Import

GeneSpring

Chip analytics

ROSETTA RESOLVER

ROSETTA LUMINATOR

FTP Export

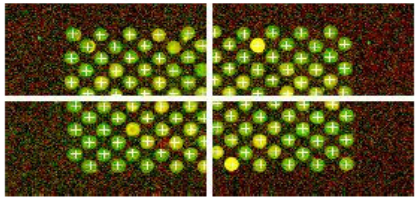
QC Metrics

Feature Extraction QC metrics

QC Report - Agilent Technologies : 2 Color CGH

Date:	Thursday, February 23, 2006 - 11:12	BG Method:	No Background
Image:	US45102826_251275011095_S01	Spatial Detrend:	On
Protocol:	CGH_44k_1005 (Read Only)	Global Adjust:	Off
User Name:	ikishawi	Dye Norm:	Linear
Grid:	012750_D_20050614	Linear DyeNorm Factor:	18.2 (Red) 28.4(Green)
FE Version:	8.5.1.1	Additive Error:	33(Red)104(Green)
		Derivative of Log Ratio Spread:	0.42

Spot Finding of the Four Corners of the Array



	Feature		Local Background	
	Red	Green	Red	Green
Non Uniform	102	53	4	4
Population	39	82	1877	2566

Net Signal statistics

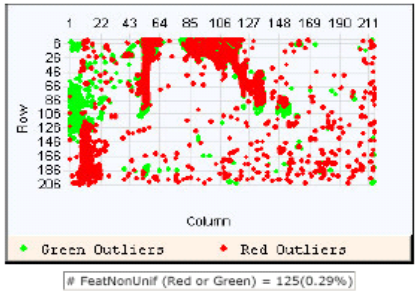
Non-Control probes:

	Red	Green
NumSat	0	1
99%	271	220
50%	68	68
1%	34	37

Negative Control Stats

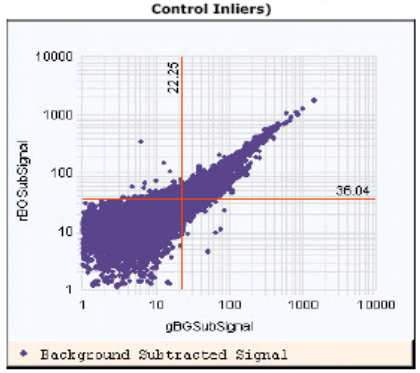
	Red	Green
Average Net Signals	32.36	45.92
StdDev Net Signals	1.93	4.45
Average BG Sub Signal	-0.03	-0.08
StdDev BG Sub Signal (BG Noise)	1.83	3.67

Spatial Distribution of All Outliers on the Array



FeatNonUnif (Red or Green) = 125(0.29%)

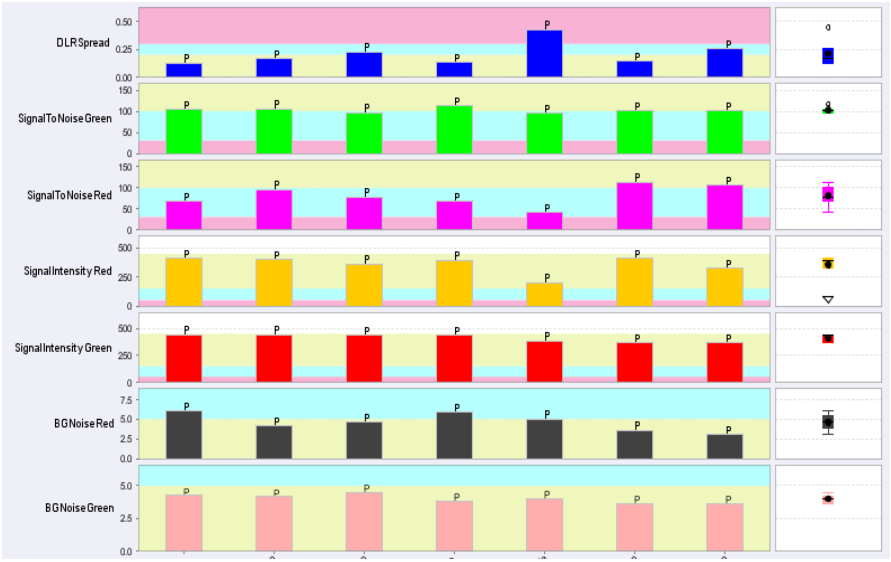
Red and Green Background Corrected Signals (Non-Control Inliers)

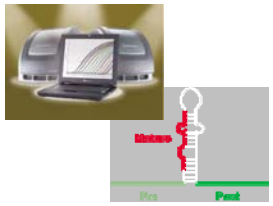


Features (NonCtrl) with BGSubSignals < 0: 189 (Red); 3238 (Green)

Local Bkg (inliers)

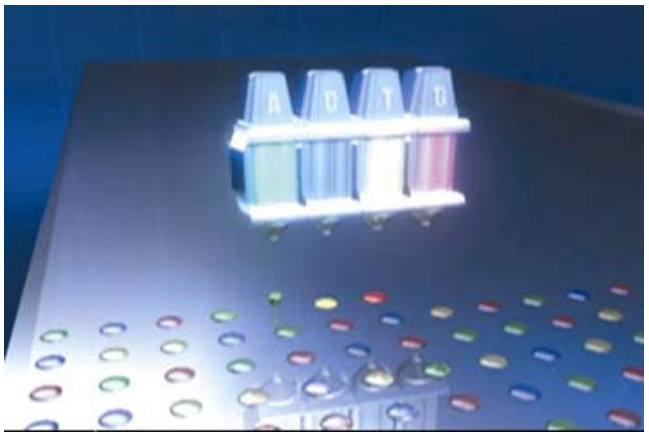
DNA Analytics QC metrics





Data quality: feature sizes and density

- Features should be far enough apart to prevent light leakage.
- Features should be big enough for perfect registration of each layer (no blurry edges), for individual feature statistics and higher confidence
- An ability to increase density while maintaining large enough features without compromising statistical significance



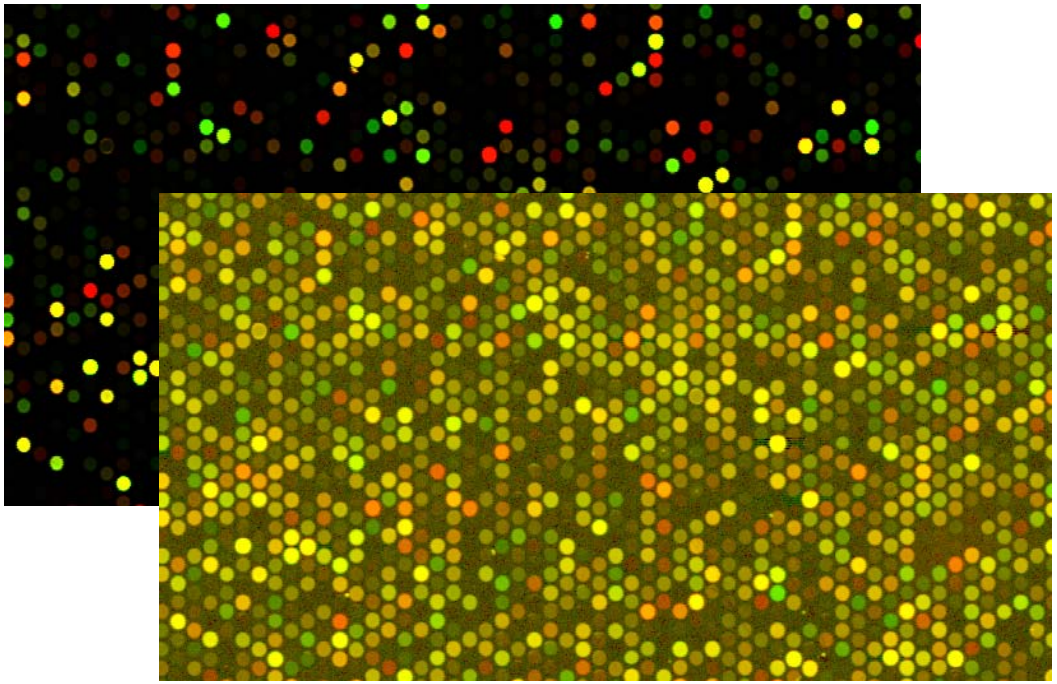
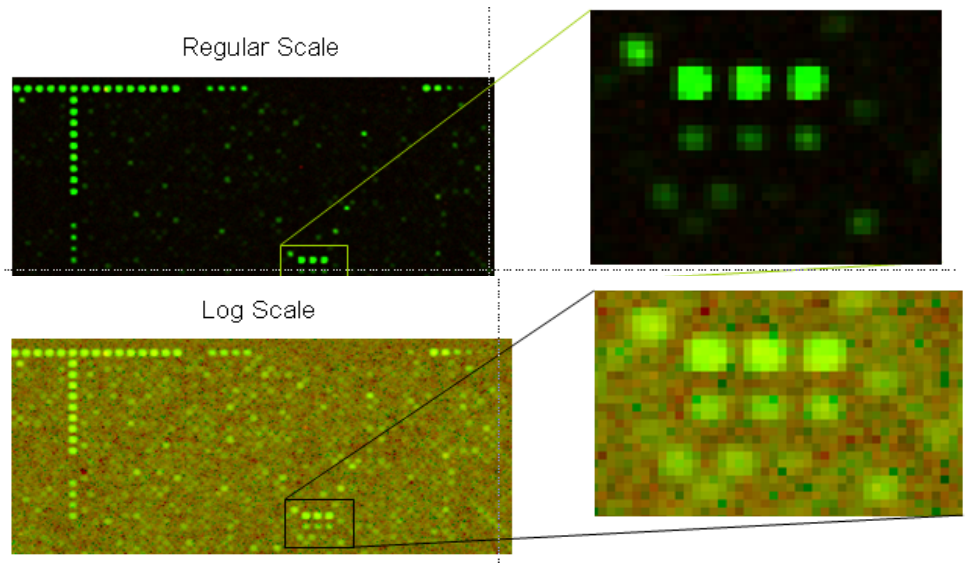
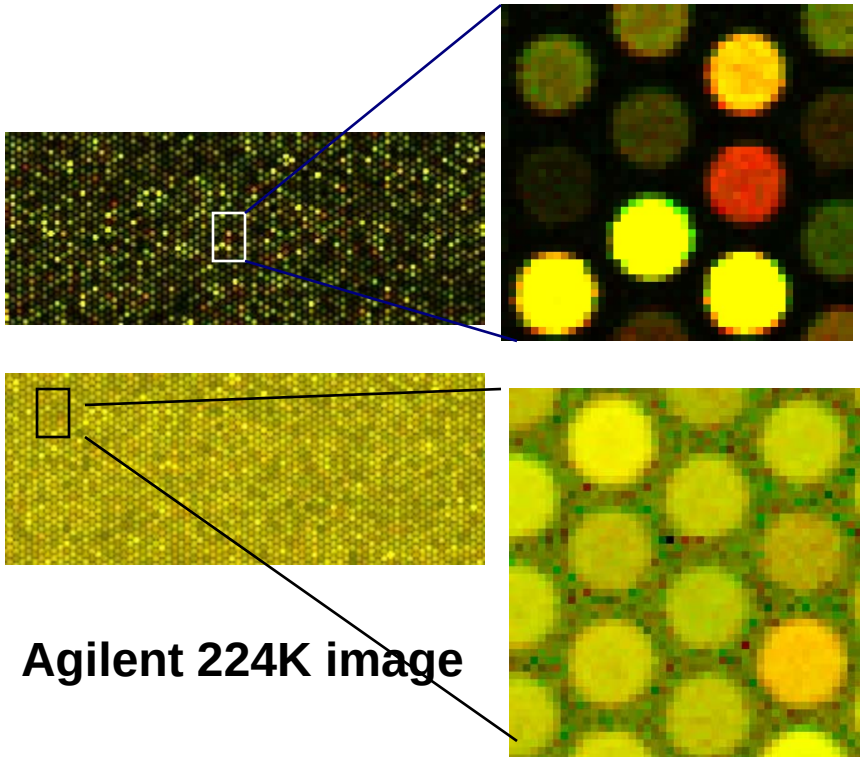


Image Value in Data quality

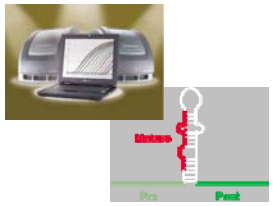
Agilent scanner at 5 micron resolution



Other array image

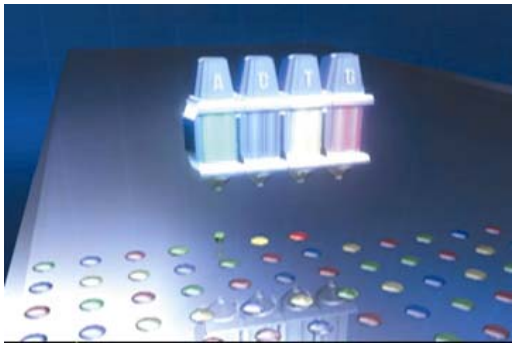


Agilent 224K image



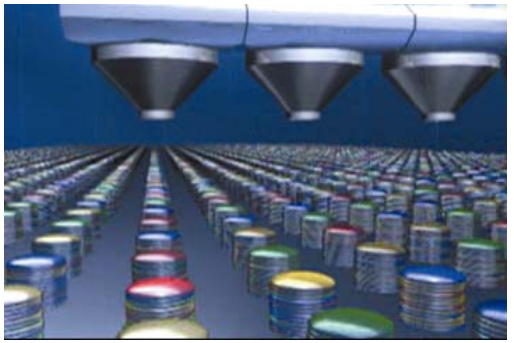
Agilent's Microarray Platform

- **Reliable inkjet printing of phosphoamidite bases**



- **Sensitivity: The chemistry reliability allows synthesis of long oligos that are highly sensitive and specific**

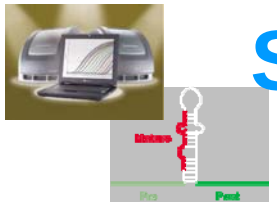
- **Flexibility of microarrays, array is defined by electronic file.**



- **Ease of implementation**

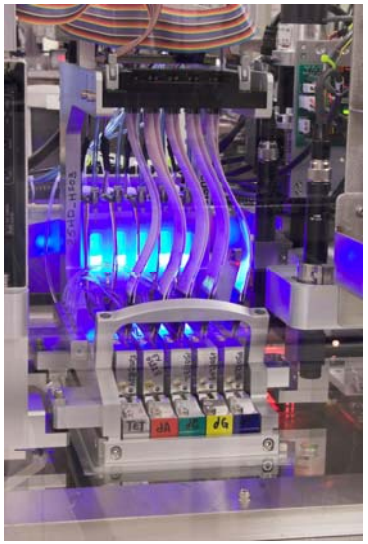
- **Probe Design: Narrow TM, Unique Probes, Self Structure, and GC content, All probes are empirically tested.**

- **Probe Fidelity can synthesize up to 200mer**

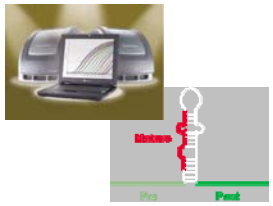


Santa Clara Manufacturing Facility

- **Manufacturing** **Process Development** **Bioinformatics**



- Industrial manufacturing – Class 10,000 clean-room
- Wired directly into eArray, allowing direct customer access to fully customizable products
- High-performance inkjet printing enables long oligo manufacturing

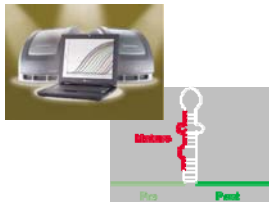


Challenges in miRNA Profiling

- Small size – difficult to label with high efficiency
- High sequence homology – difficult to design probes with high specificity
- Presence of larger RNAs with highly homologous sequences
- Expressed with large dynamic range – difficult to avoid compression
- Growing & changing database

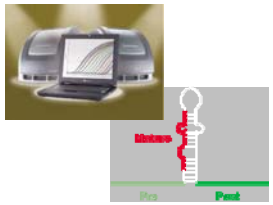
miRNA	Sequence	#NT
hsa-let-7a	ugagguaguagguuguauaguu	22
hsa-let-7b	ugagguaguagguugugugguu	22
hsa-let-7c	ugagguaguagguuguaugguu	22
hsa-let-7d	agagguaguagguugcauaguu	22
hsa-let-7e	ugagguaggagguuguauaguu	22
hsa-let-7f	ugagguaguagauuguauaguu	22
hsa-let-7g	ugagguaguaguuuguacaguu	22
hsa-let-7i	ugagguaguaguuugugcuguu	22

Sanger
miRBase 12.0



miRNA Arrays:

- Human miRNA Microarray, v2.0:
  - 799 distinct probe sets (723 human and 76 human viral)
- Human miRNA Microarray, v1.0:
  - 450 human distinct probe sets



Direct and Sensitive miRNA Profiling

Hui Wang, PhD
Senior Scientist
Agilent Technologies

METHOD

Direct and sensitive miRNA profiling from low-input total RNA

HUI WANG, ROBERT A. ACH, and BO CURRY
Agilent Technologies, Inc., Agilent Laboratories, Santa Clara, California 95051, USA

ABSTRACT

We have developed a sensitive, accurate, and multiplexed microRNA (miRNA) profiling assay that is based on a highly efficient labeling method and novel microarray probe design. The probes provide both sequence and size discrimination, yielding in most cases highly specific detection of closely related mature miRNAs. Using a simple, single-vial experimental protocol, 120 ng of total RNA is directly labeled using Cy3 or Cy5, without fractionation or amplification, to produce precise and accurate measurements that span a linear dynamic range from 0.2 amol to 2 fmol of input miRNA. The results can provide quantitative estimates of the miRNA content for the tissues studied. The assay is also suitable for use with formalin-fixed paraffin-embedded clinical samples. Our method allows rapid design and validation of probes for simultaneous quantitative measurements of all human miRNA sequences in the public databases and to new miRNA sequences as they are reported.

Keywords: miRNA profiling; microarray; RNA labeling; probe design; microRNA

RODUCTION

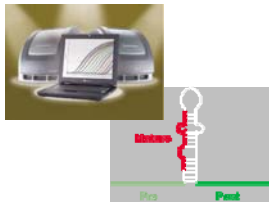
oRNAs (miRNAs) are a class of small single-stranded oding RNAs [19–30 nucleotides (nt)] that have been d in animals, plants, and viruses, with over 400 ified in humans (Bartel 2004; Griffiths-Jones 2004; ore and Haley 2005; Griffiths-Jones et al. 2006; Kim Nam 2006). MicroRNA genes are transcribed as pri- NAs, which are then processed to the shorter hairpin ed pre-miRNAs (~70–90 nt) before they are cleaved to the mature single-stranded miRNAs (~22 nt) (Bartel ; Zamore and Haley 2005; Kim and Nam 2006). As of a multiprotein RNA-induced silencing complex, the NAs repress translation by forming imperfect base pairing with the 3′ untranslated region of target messenger RNAs (mRNAs). Bioinformatics and cloning studies have estimated that miRNAs may regulate 30% of all human et al. 2005). Recent studies RNA expression patterns are or types (Calin et al. 2004; l. 2006; Esquela-Kerscher and

The ardent interest in profiling miRNA expression has led to developments in Northern blot (Cummins et al. 2006), cloning (Lau et al. 2001), PCR (Chen et al. 2005), bead-based (Lu et al. 2005), SAGE-based (Cummins et al. 2006), and microarray-based methods (Babak et al. 2004; Barad et al. 2004; Calin et al. 2004; Liu et al. 2004; Nelson et al. 2004; Baskerville and Bartel 2005; Liang et al. 2005; Lim et al. 2005; Shingara et al. 2005; Castoldi et al. 2006). The ideal method would require submicrogram quantities of total RNA, have minimal sequence bias, be easy to multiplex, and involve simple experimental protocols. Microarray-based assays offer an efficient method for measuring the expression profile of large numbers of miRNAs simultaneously. However, the small size and high sequence homology of miRNAs present major challenges to sample labeling and microarray probe design.

Here we present a novel miRNA assay employing simple high-efficiency direct labeling of submicrogram quantities of total RNA, without amplification or size fractionation. Our labeling protocol has little sequence bias, and our in situ synthesized DNA microarray probes (Hughes et al. 2001) are both sequence and size selective for mature miRNAs. The enzymatic labeling attaches a single fluorophore-labeled nucleotide to the 3′ end of each miRNA with high yield and minimal sample manipulation. Hybridization to the microarray is carried out under conditions that result in near-equilibrium binding and high (>29%) hybridization yields for most miRNAs. The assay is easy to perform, has

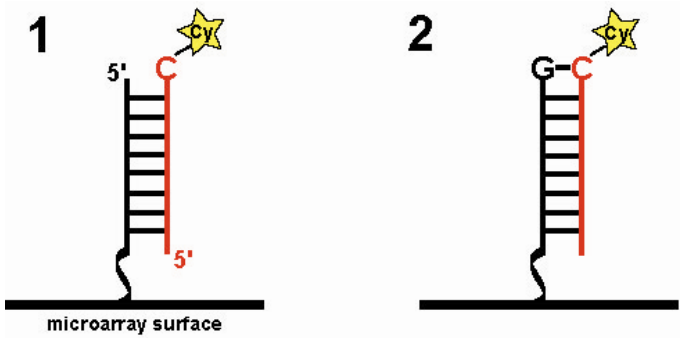
Agilent Technologies, Inc., Agilent ...leward, Santa Clara, CA 95051, USA; ...408) 553-2161.

...nt. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.234507.



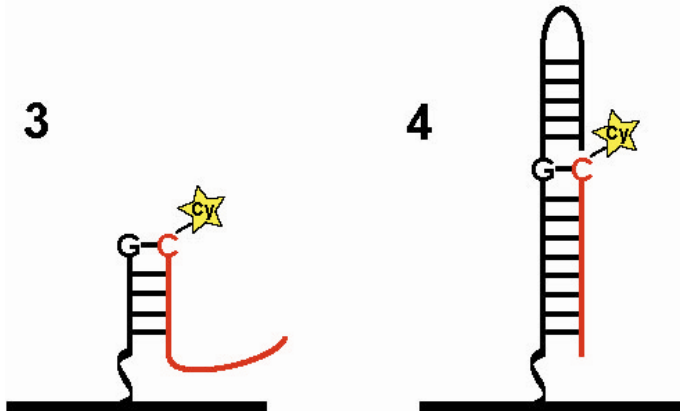
miRNA Probe Design Strategy

1. Start design with full-length miRNA-probe sequence, attached to a stilt sequence.



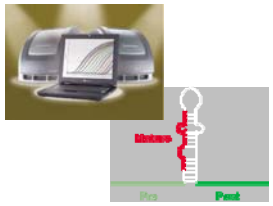
2. Utilize the **C** incorporated during labeling for additional **G-C** base pair on 3'end of miRNA to increase stability

3. Sequentially shorten target-probe base pairing from 5' end of miRNA during preliminary Tm balancing by calculation.



4. Incorporate hairpin structure on probes to increase size specificity and probe:target stability.

FINAL STEP:
Select Tm-balanced probes for each miRNA empirically using microarray data.



Work Flow

Total RNA
(100 ng)

↓ **Direct Label with Cy-dye**

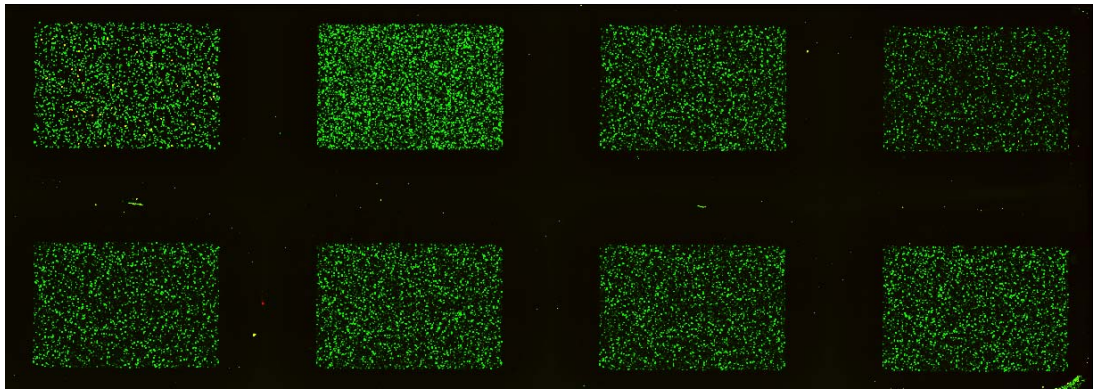
Labeled RNA

↓ **Hybridize**

miRNA Profile

8-pack

100ng sample input
Direct probe labeling
High specificity & sensitivity



Specificity to Distinguish Homologous

miRNAs

Probes	7a	7b	7c	7d	7e	7f	7g	7i
7a	100	10	51	3	1	5	2	0
7b	0	100	1	0	0	0	7	0
7c	6	70	100	1	0	0	3	0
7d	1	2	1	100	39	0	3	0
7e	4	1	1	2	100	0	1	0
7f	62	3	5	1	0	100	1	0
7g	1	0	0	0	0	0	100	1
7i	0	1	0	0	0	0	4	100

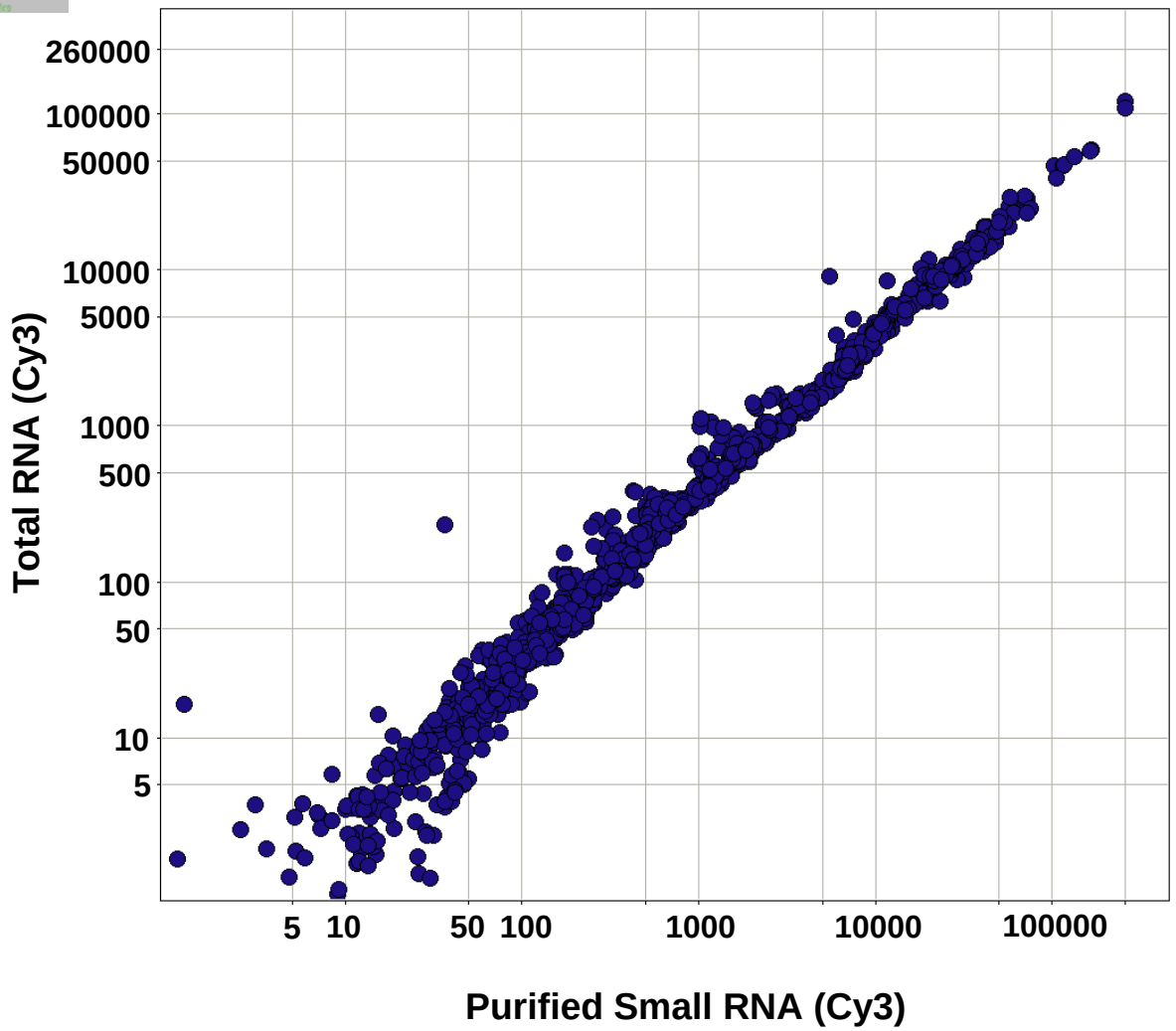
85 - 100%
70 - 84%
55 - 69%
40 - 54%
25 - 39%
10 - 24%
5 - 9%
0 - 4%

Grey Number

hsa-let-7 family

miRNA	Sequence	Length (nts)
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGU**U**	22
hsa-let-7b	UGAGGUAGUAGGUUGU**G**U**G**GU**U**	22
hsa-let-7c	UGAGGUAGUAGGUUGUAU**G**GU**U**	22
hsa-let-7d	**A**GAGGUAGUAGGUUG**C**AUAGU	21
hsa-let-7e	UGAGGUAG**G**AGGUUGUAUAGU	21
hsa-let-7f	UGAGGUAGUAG**A**UUGUAUAGU**U**	22
hsa-let-7g	UGAGGUAGUAG**U**UUGUA**C**AGU	21
hsa-let-7i	UGAGGUAGUAG**U**UUGU**GCU**GU	21

Direct miRNA Measurement From Total RNA

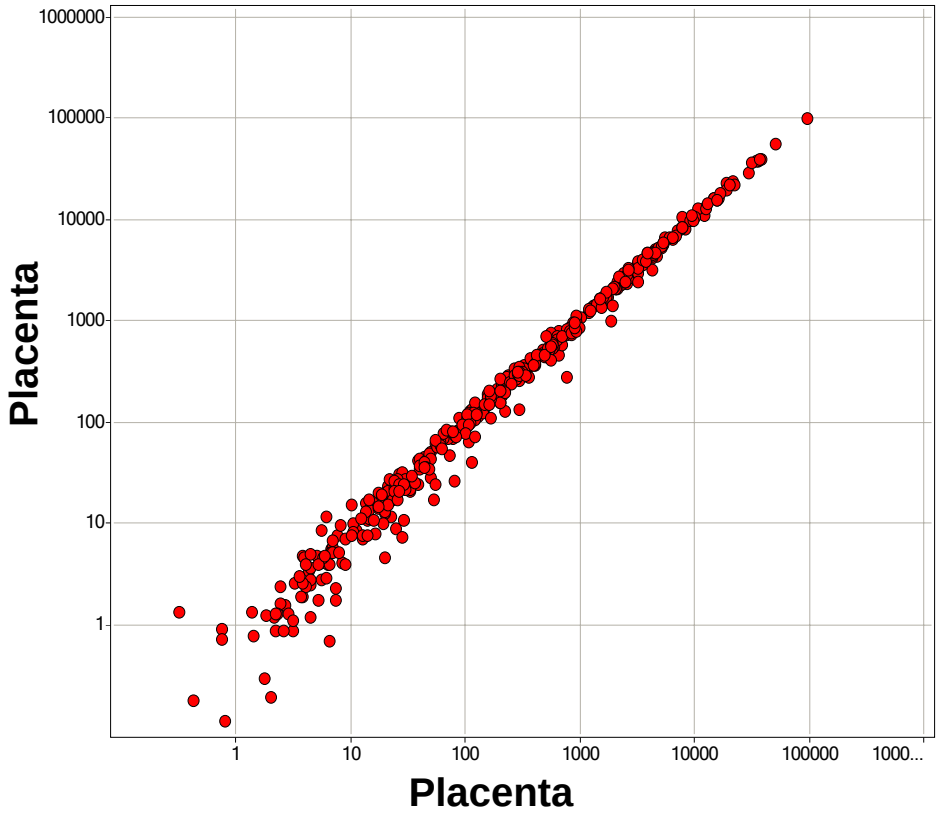


Data shown are background-subtracted signals with no filtering or normalization.

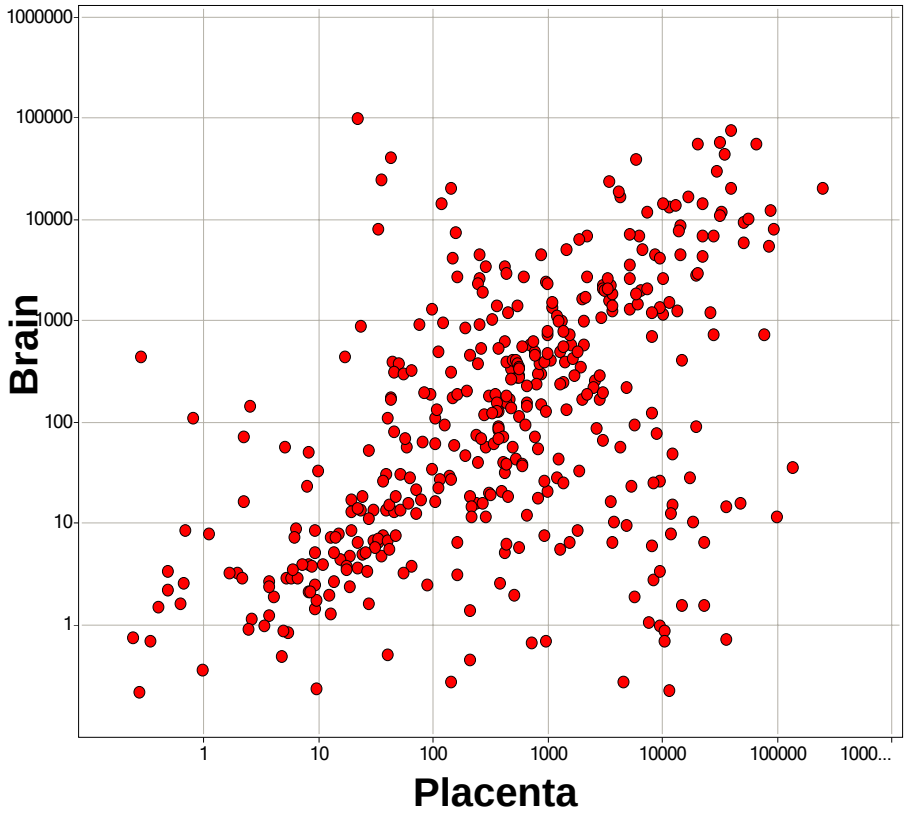
Each miRNA has signals from multiple probes.

miRNA Profiles Using 100ng Total RNA

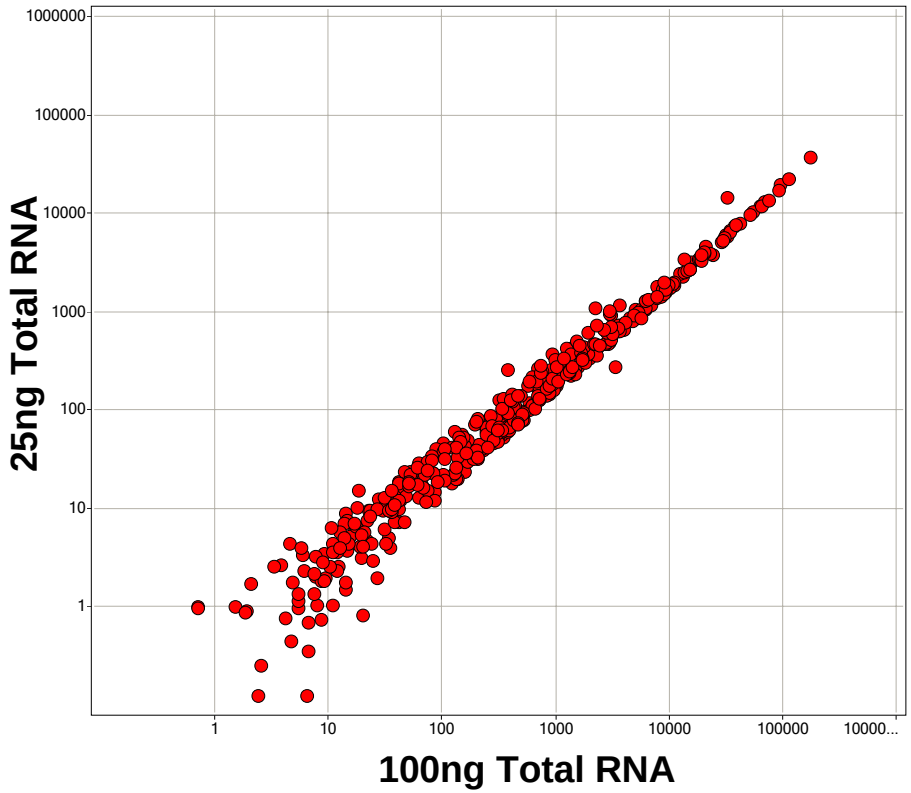
Reproducibility

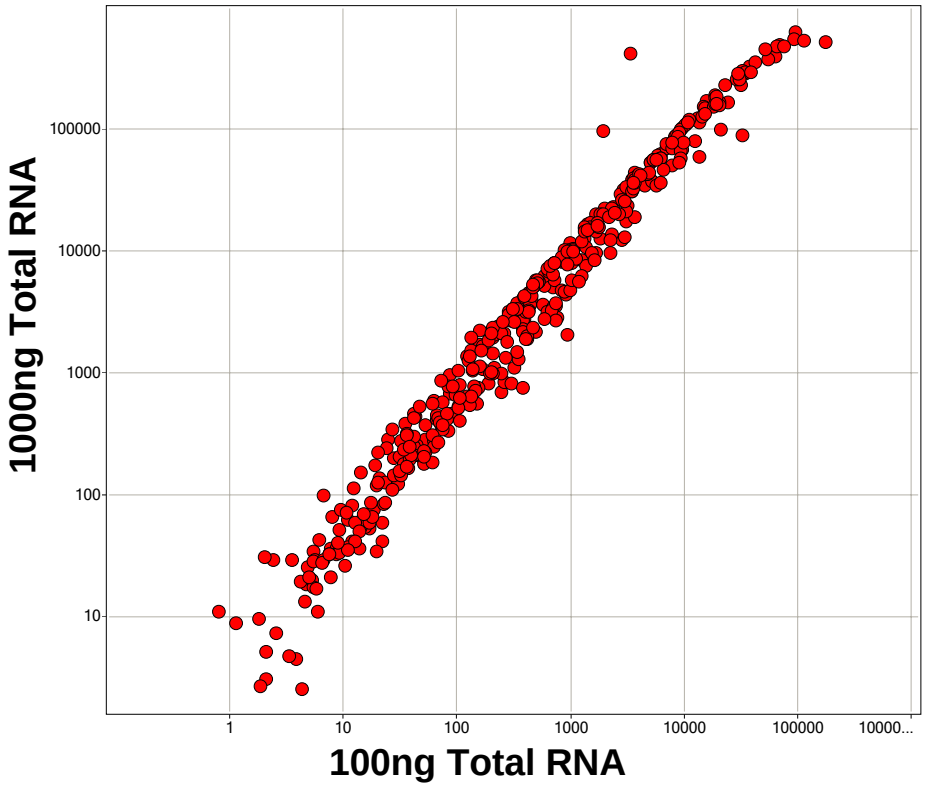


Differential Expression

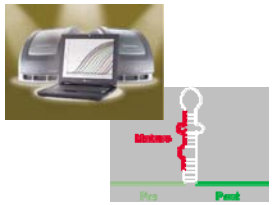


miRNA Profiles Independent of Sample Input



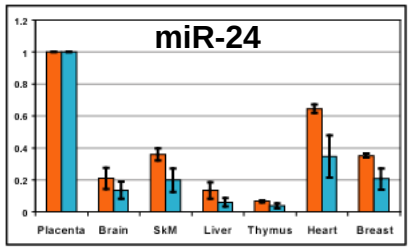


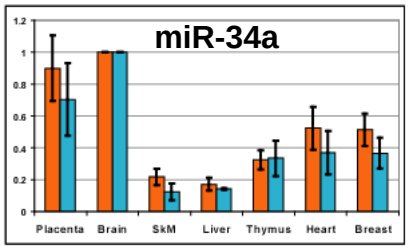
Consistent miRNA profile for low (25ng) vs high (1000ng) total RNA input

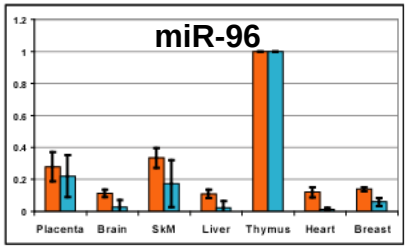


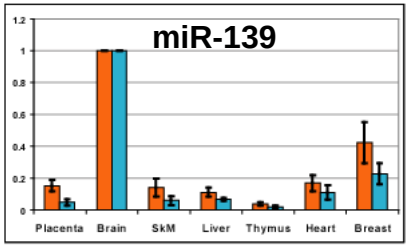
Correlation between microarray and quantitative RT-PCR results

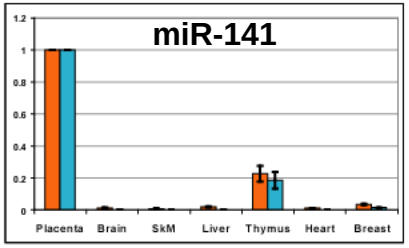
Quantitative RT-PCR (qPCR) reactions (orange bars) and microarray hybridizations (blue bars) were run in quadruplicate for each miRNA species.

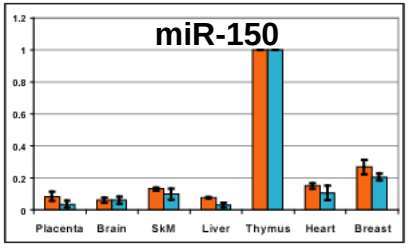


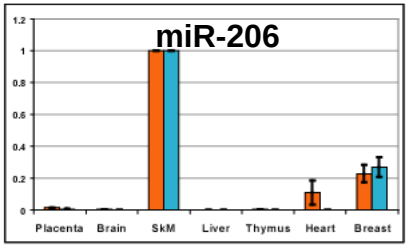


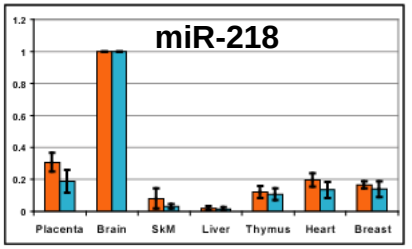


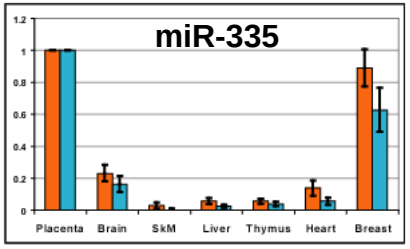


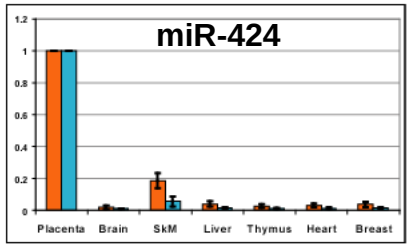






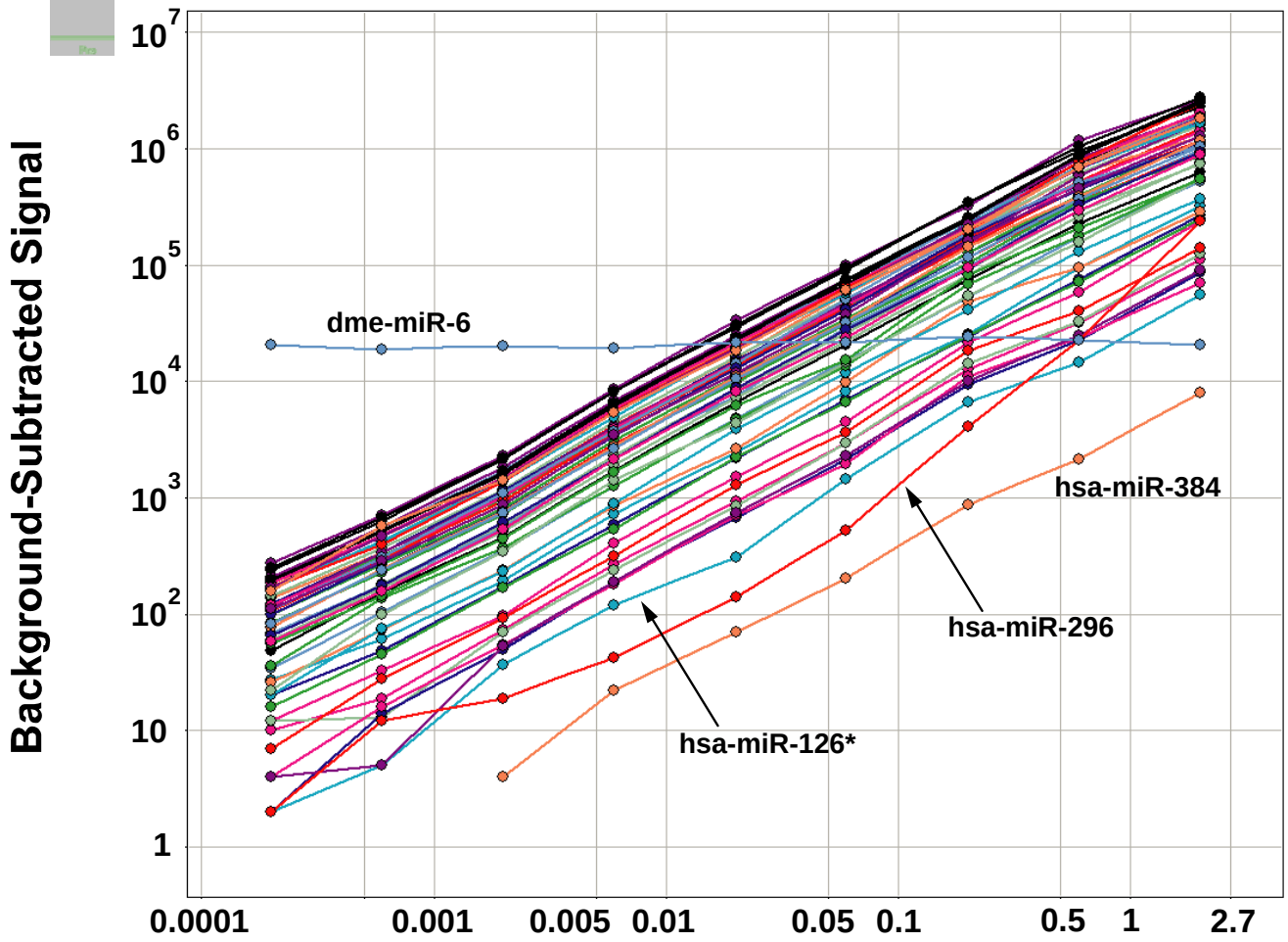






qPCR Data Microarray Data

10^4 Linear Dynamic Range



Synthetic miRNAs were selected for typical and atypical sequence representations.

Lowest and highest calculated Tms are represented.

Most miRNAs are detectable at the 0.2αmol-2fmol range.

Platform Features, Benefits & Advantages

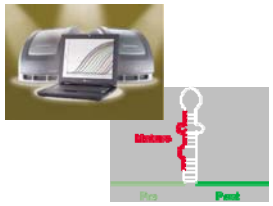
Feature	Benefit	Advantage to Investigator
8 x 15K format	Process multiple samples on the same slide	Reduce costs Run parallel experiments
Optimized probe design and direct labeling	Greater sensitivity and specificity	Detect low abundance miRNAs Detect highly homologous miRNAs
Small sample input	Ability to work with precious samples	Analyze new sample types Conserve precious samples
Use of total RNA	Does not require size separation	Does not introduce any miRNA isolation bias Easy to use Reduce hands-on time
Broad dynamic range	Detect all possible miRNAs in sample	Comprehensive miRNA profiling
Compatible with standard microarray platform	Correlate miRNA profiles with GE and CGH data	Requires only single capital investment Enables integrated genomics

Response from Early Access Customer

We have shown for the first time that let-7 expression is frequently reduced in lung cancers and that alterations in the miRNA expression may have a prognostic impact on the survival of surgically treated lung cancer patients. Agilent miRNA arrays give us the comprehensive miRNA expression profile with excellent performance on sensitivity and accuracy. I expect that the studies of Agilent miRNA array may ultimately provide a foundation for a new paradigm of the involvement of miRNA in human oncogenesis.

Dr. Takashi Takahashi
Professor of Oncology
Molecular Carcinogenesis

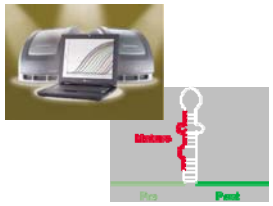
Nagoya University



Stratagene QPCR MicroRNA Studies

Featuring:

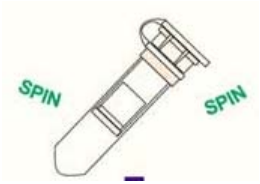
High-Specificity miRNA QRT-PCR Detection Kit



Purification of miRNA

Challenges in small RNA Purification

Spin colums

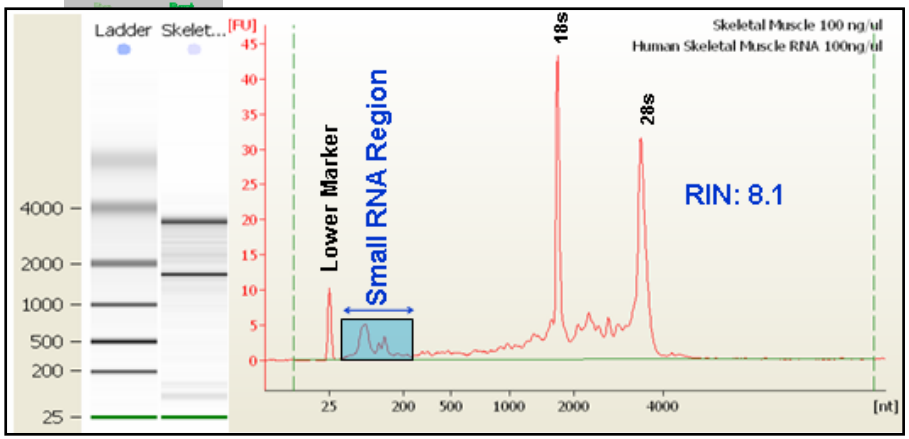


- Retention of small RNAs on standard columns not sufficient: → Low yield of small RNAs
- Specialized solutions available: **miRACLE™ miRNA Isolation kit**

- Enrichment of miRNA species for increased sensitivity using specialized solutions like miRACLE™
- Usually Phenol-Chloroform based clean up to avoid loss of small RNAs
- Alternative methods like SideStep™ (Cells-to-PCR) available

Purification of miRNA

Sample Verification – Bioanalyzer Small RNA kit



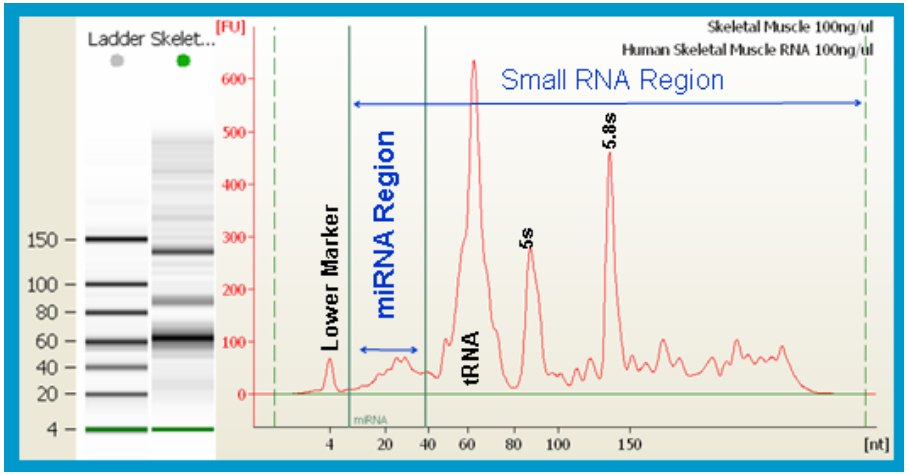
RNA 6000Nano kit

RNA 6000 Nano Kit

Size range: 25-6000nt

Results: Integrity, Total RNA amount, gDNA contamination

Small RNA Kit



NEW! Small RNA Kit
Size range: 6-150nt

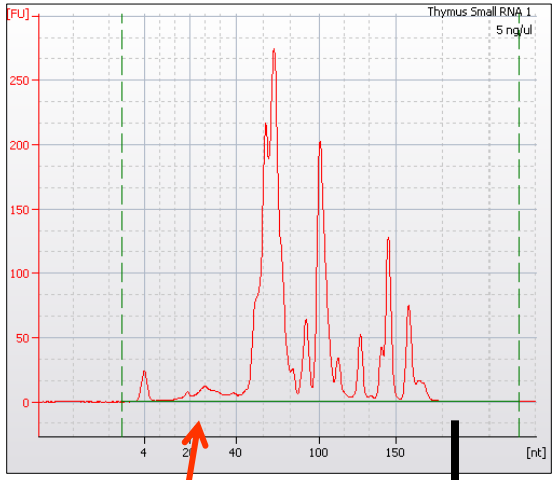
Results: miRNA amount, Ratio and amount of other Small RNA

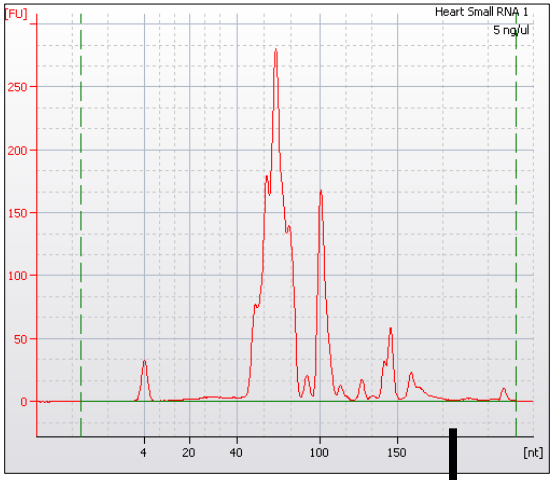
Purification of miRNA

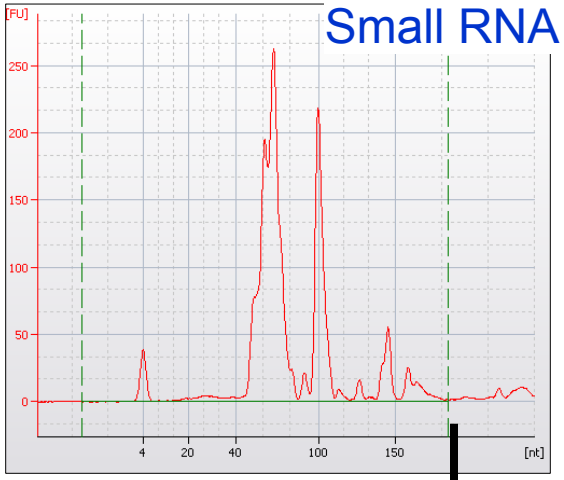
Sample Verification – Bioanalyzer Small RNA kit

Small RNA

Pre-purified small RNA (0-150 nt)

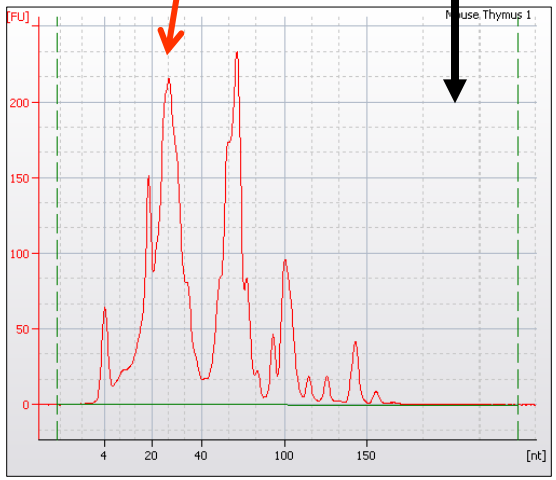


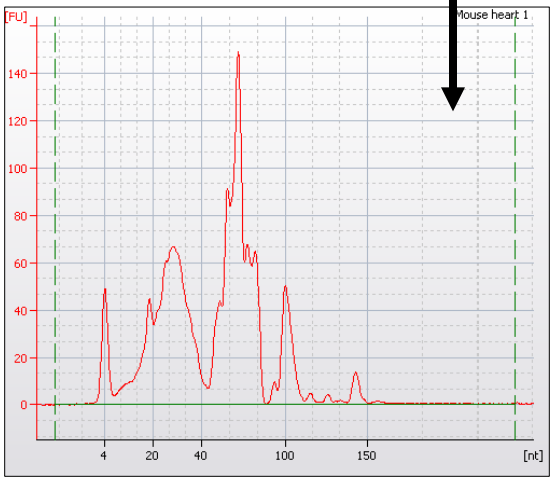


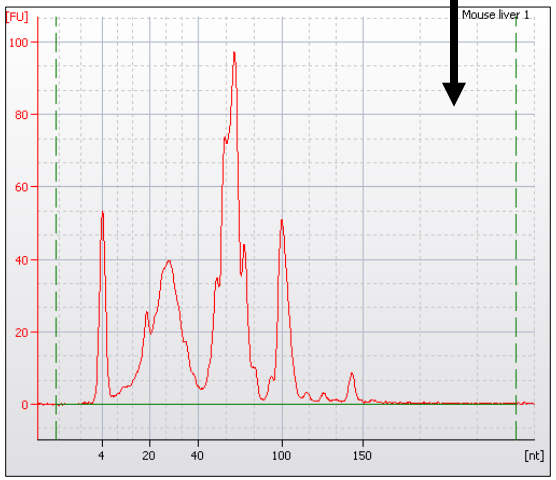


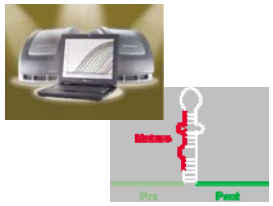
miRNA Peak

enriched miRNA fraction





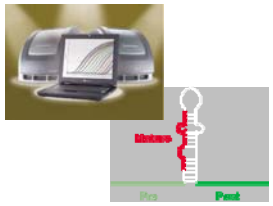




miRNA Detection
Challenges in QPCR

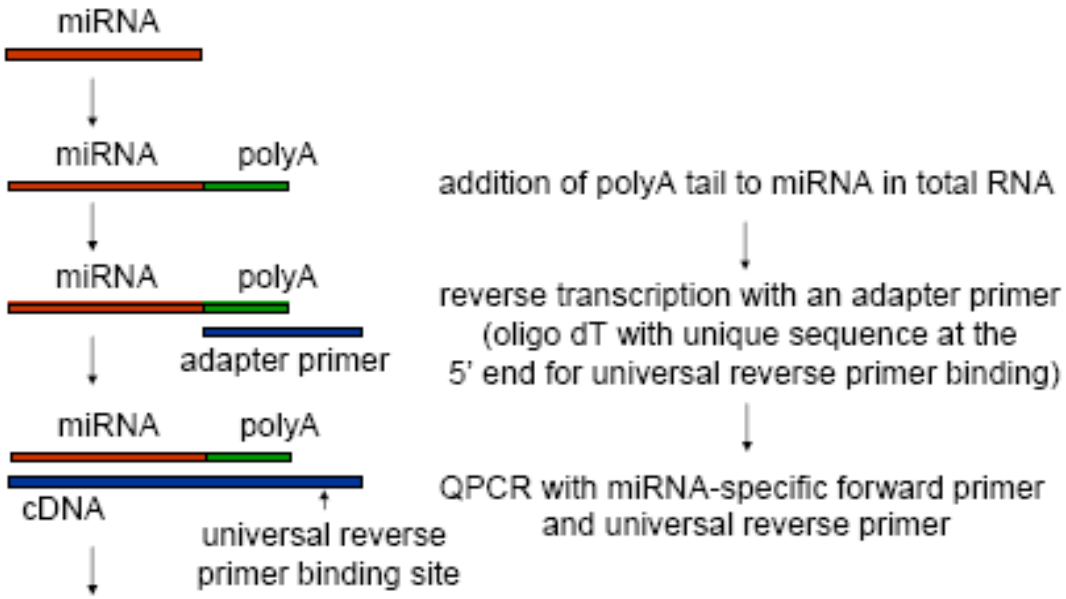
Design challenges:

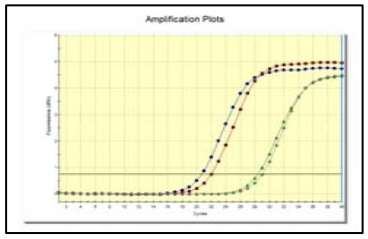
- Sequence length of miRNAs is very short
- Lack of common nucleotide sequence to be used as priming site
- High sequence homology between different miRNAs
- Presence of different forms of miRNA in a cell

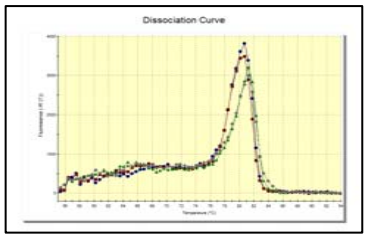


QRT-PCR Detection

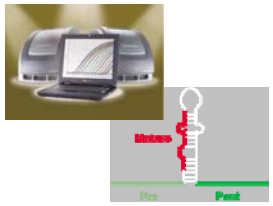
High-Specificity miRNA QRT-PCR Detection Kit







- **High-Specificity miRNA QRT-PCR Detection Kit**
  - **miRNA 1st Strand cDNA Synthesis Kit**
  - **High-Specificity miRNA QPCR Core Kit**



Specificity of High-Specificity miRNA QRT-PCR Detection Kit

Discrimination between all let-7 family members using miRNA-specific PCRprimers.

miRNA-specific assay

miRNA synthetic templates	let-7a	let-7b	let-7c	let-7d	let-7e	let-7f	miR-98	let-7g	let-7i
let-7a	100	0	0	0	0.7	0.2	0	0	0
let-7b	0.0	100	0.6	0	0	0	0	0	0
let-7c	0.8	0.1	100	0	0	0	0	0	0
let-7d	0.1	0	0	100	0	0	0	0	0
let-7e	0.6	0	0	0	100	0	0	0	0
let-7f	0.2	0	0	0	0.1	100	0	0	0
miR-98	0	0	0	0	0	0	100	0	0
let-7g	0	0	0	0	0	0	0	100	0
let-7i	0	0	0	0.1	0	0.1	0	0.1	100

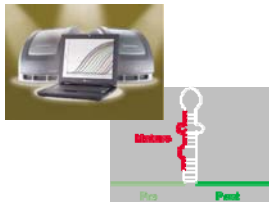
% relative detection

0nt 1nt 2nt 3nt >3nt

number of mismatches

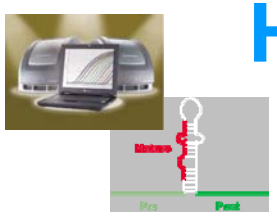
- **The same number of let-7 miRNA (10^{10} miRNA synthetic templates) were converted to cDNA and detected with each of the let-7 miRNA-specific primers (miRNA-specific assay).**

- Detection with each of the let-7 miRNA-specific primers (miRNA-specific assay)
- Copy number of the matching primer and template was 100%.
- Copy number of a non-matching primer and template determined as a percentage of the matching primer and template (% relative detection)
- The number of mismatched nucleotides is indicated in color as shown in the key (number of mismatches).



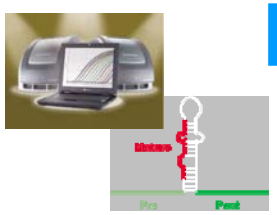
High-Specificity miRNA QRT-PCR Detection Kit Features

- Detects mature miRNA in 3 hours
- As little as 15 ng of total RNA input
- Variety of sample types
  - Lysed cells
  - Total RNA isolated from tissues
  - Cell cultures
  - FFPE tissues
- Sensitive detection down to 10 copies
- Single-nucleotide discrimination
- Detection of up to 6,000 different miRNA in a single sample preparation



High-Specificity miRNA QRT-PCR Detection Kit Features

Feature	High-Specificity miRNA QRT-PCR Detection Kit
Sensitivity	33 copies in 3.3ng total RNA (10 copies/ng)
Template (lowest)	30pg total RNA/600-6,000 miRNA
Template (recommended)	250-1000ng/600-6,000 miRNA
Linearity	7 logs
# human assays	50 human



High-Specificity miRNA Detection Kit

- 50 miRNA-specific primers based on human miRNA

- selection was based on differential expression in cancer and during development most will also detect mouse and rat miRNA

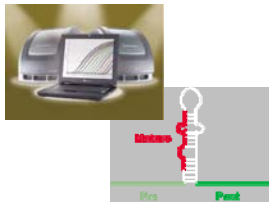
human	mouse	rat
50	41 (miR-106a)	40 (miR-15a, miR-106a)

- will be adding another 8 miRNA-specific primers to the list

- human and mouse U6 primers for normalization

- primer design rules will be available to customers on our website

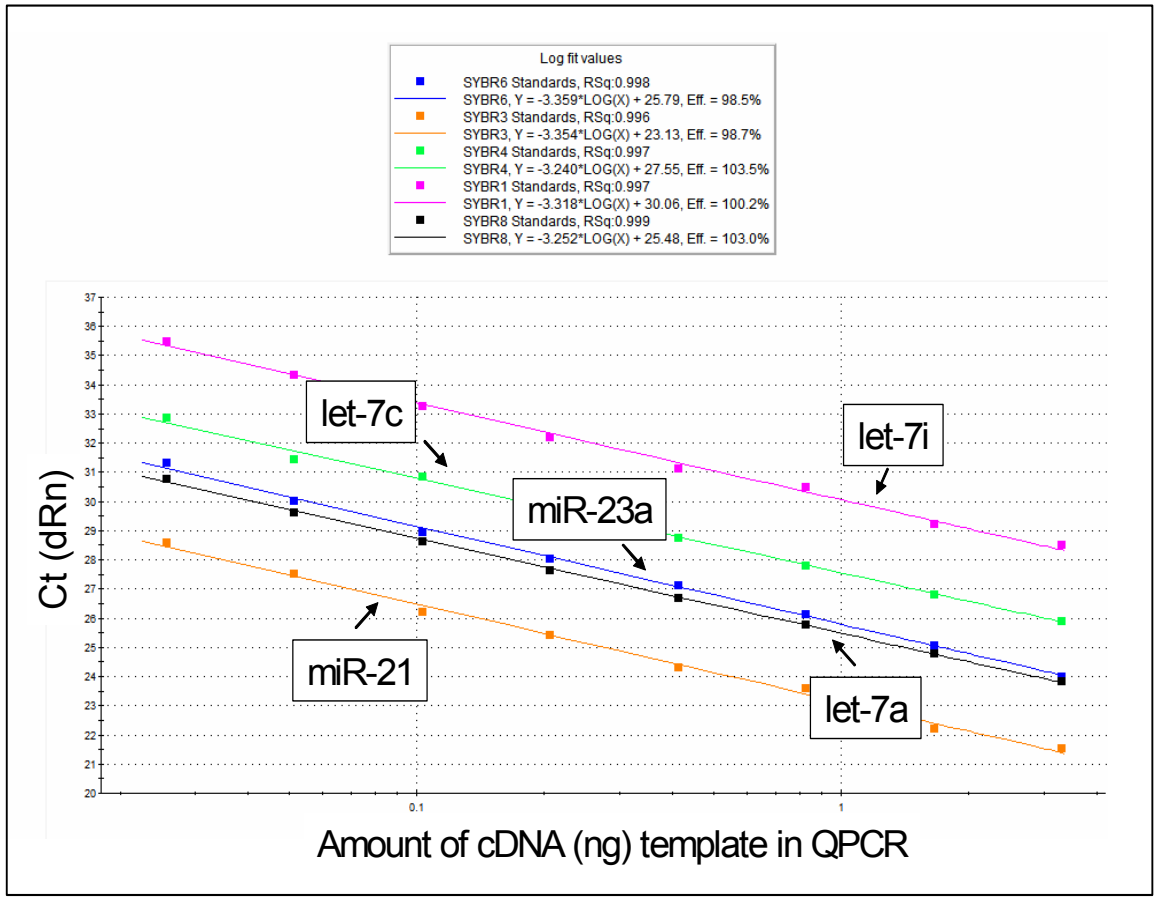
lot number from RT adapter primer has to be input to access primer design rules



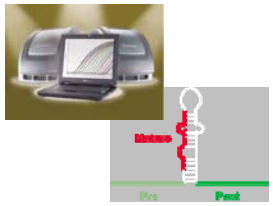
Kit Performance: Assay Linearity

- cDNAprepared from 1ug HeLa total RNA and using varying amounts as template in QPCR.

- Five different miRNA of varying abundance, let-7a, let-7c, let-7i, miR-21 and miR-23a, were detected.



- 25 pg – 3.3 ng cDNA input. Detection of miRNAs of varying abundance
- Linearity over 7 logs (after Poly A tailing and cDNA synthesis)



Detection of miRNA Expression

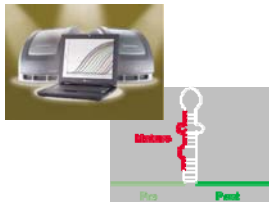
Breast Cancer Biomarker study

Introduction

- Preserved breast tumor tissue samples were obtained from ER positive and PR positive pre-menopausal women
- Women were treated post-surgery with tamoxifin. Clinical data indicated a wide range of mortalities (full responder, partial responder, non responder).
- All samples were from Caucasian women diagnosed with infiltrating duct carcinoma and had been preserved 8 years prior to RNA extraction (FFPE tissues)

Purpose

Retrospective study to demonstrate our High-Specificity miRNA QRT-PCR Detection kit successfully detects miRNA isolated from FFPE samples.



Detection of miRNA Expression in Breast Cancer

- Scott Basehore
- Natalia Novoradovskaya

Sample Preparation

Deparaffinize and digest FFPE tissues

Pulverize frozen tissues in liquid nitrogen

homogenize in Absolutely RNA lysis buffer: Lysate can be stored at -80°C

separate RNA and gDNA with acidified phenoll

Nucleic Acid Isolation and QC

Isolate total RNA using Absolutely RNA® kit

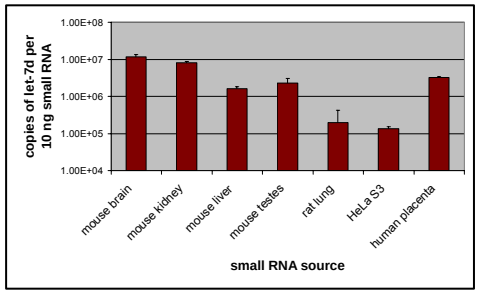
Isolate gDNA from acidified phenol phase

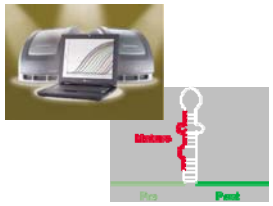
Isolate miRNA from aqueous phase

Results:

Ct (dRn)

RNA sample, 50 ng	B2M	GAPDH	HER2	PGR	Ki67	BIRC5
Frozen breast, normal	18.89	20.01	30.57	30.47	33.83	48.7
FFPE breast cancer 1	20.63	23.41	29.6	28.85	34.86	No Ct
FFPE breast cancer 2	20.22	23.23	31.48	26.02	32.33	36.12
FFPE breast cancer 3	20.43	24.82	30.19	36.13	37.3	No Ct
UHRR	19.44	16.35	32.96	28.01	25.75	26.24



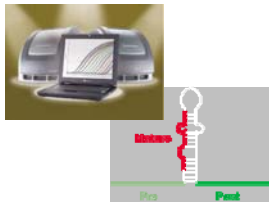


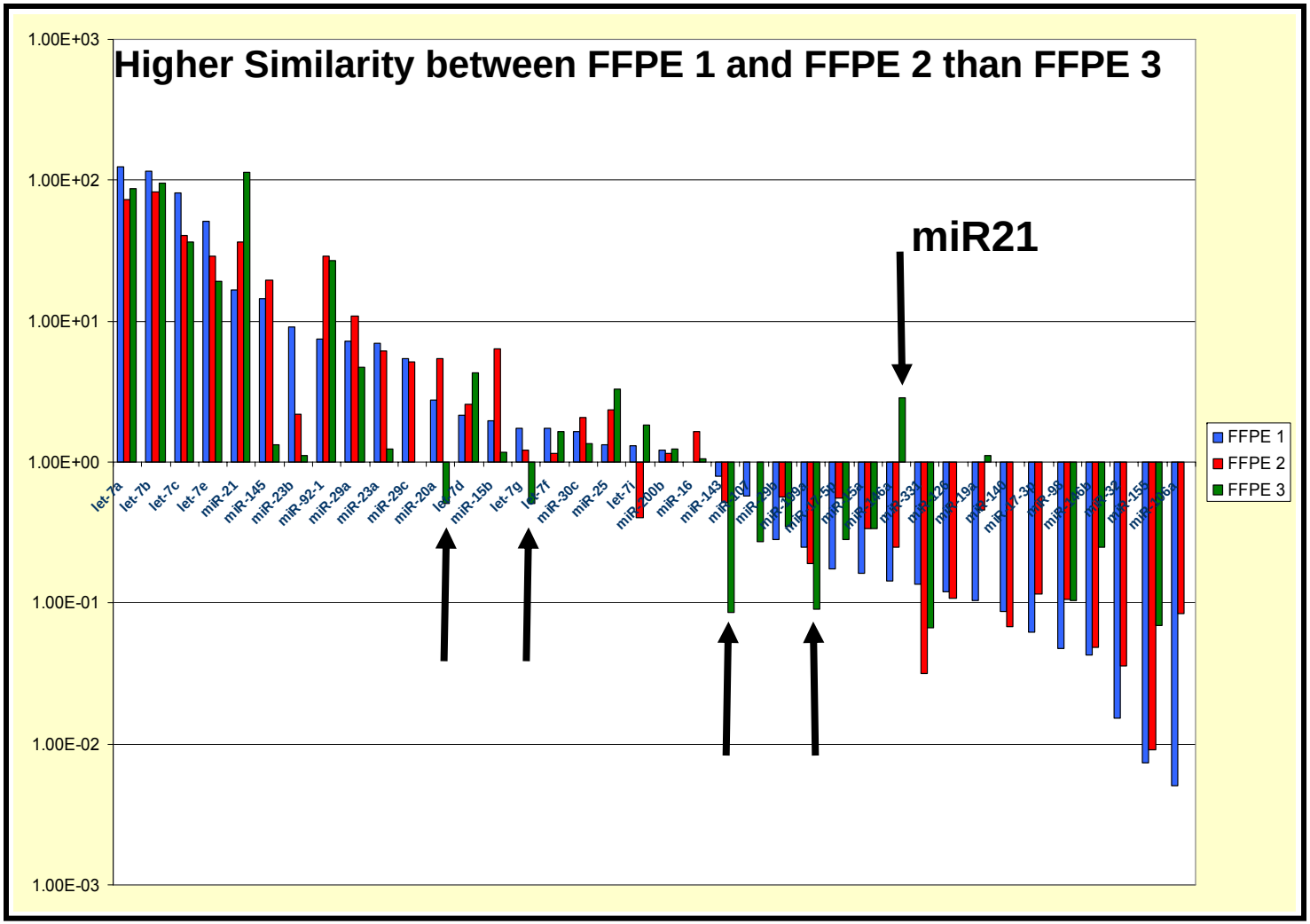
Detection of miRNA Expression in Breast Cancer

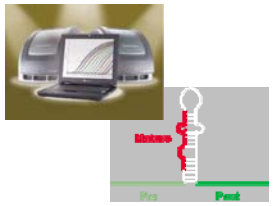
FFPE	Age	Differen-tiation Grade	TNM	Stage	No. Pos. lymph nodes	No of analyzed lymph nodes	Tumor size
1	47	grade 2	T2N0M0	IIA	0	6	5 cm
2	42	grade 2	T1N0M0	IIA	0	5	1 cm
3	51	grade 2-3	T2N1M0	IIB	2	7	3 cm

FFPE	Location of distant metastasis	Relapse (Y/N) w/in observation time	Disease-free survival (mos)	Death?	Death from main disease
1	no	no	84 mos	no	no
2	no	no	65 mos	no	no
3	lung, bone	yes	54 mos	yes	yes

Detection of miRNA Expression in Breast Cancer

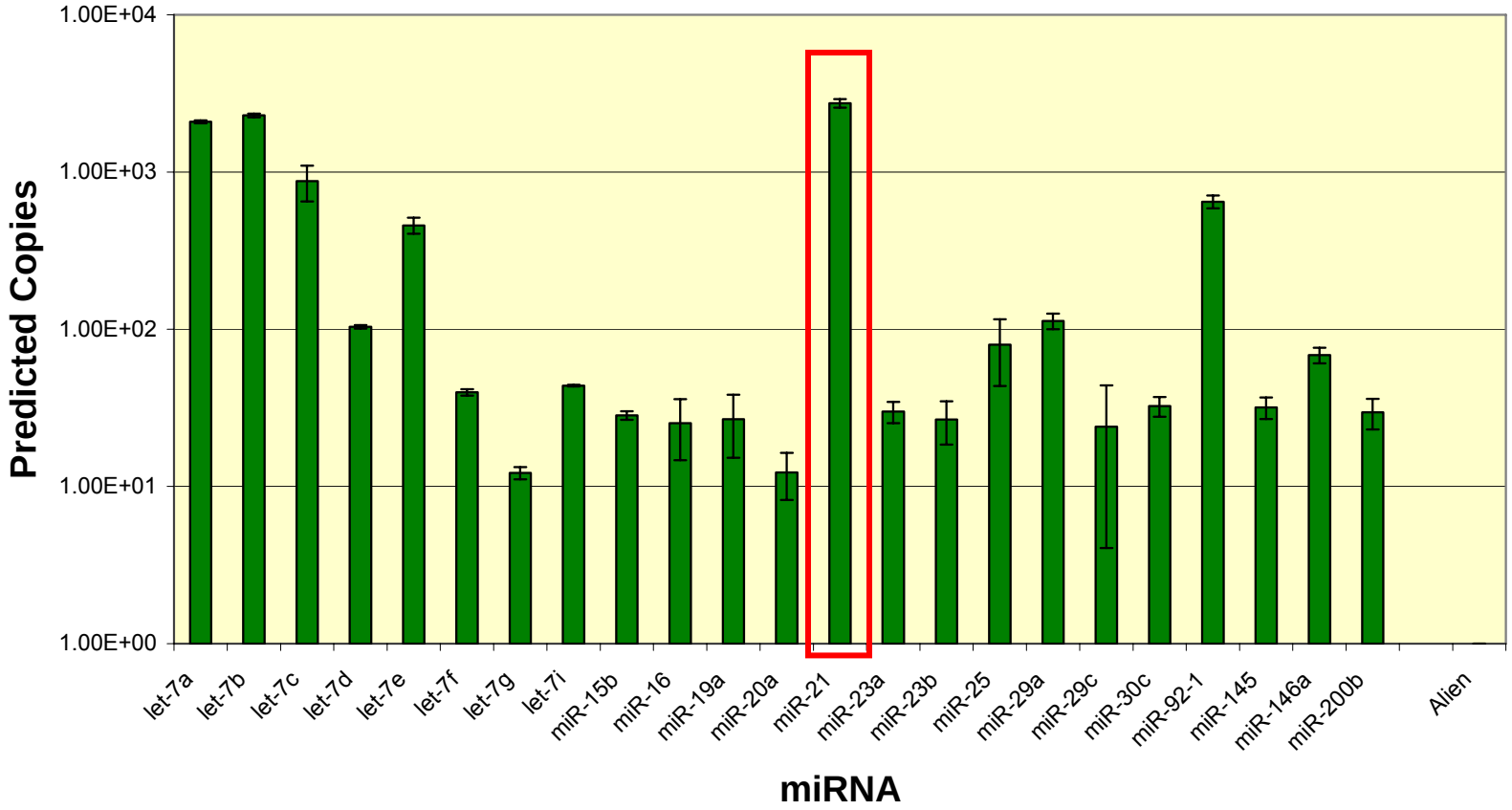


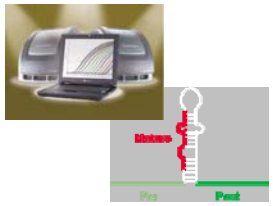




Detection of miRNA Expression in Breast Cancer

miRNA Profiling in Breast Cancer
FFPE Tissue RNA





Detection of miRNA Expression in Breast Cancer

Conclusion:

- mRNA and miRNA RNA isolated using the Absolutely RNA FFPE kit from FFPE tissues that had been preserved 8 years were of high quality and suitable for QPCR analysis

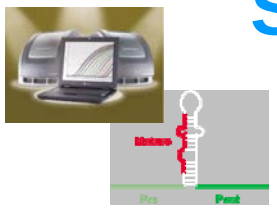
- Fold differences of miRNA levels during cancer progression from stage I to IIA and to IIB show significant up-regulation of miR-21. This is in agreement with various published results on solid tumors.*

*Volinia, S., et al (2006) Proc Natl Acad Sci 103:2257-2261

miRNA Forward Primers Relative to Disease

Types of Cancer	
Brain	miR-21
Bladder	miR-21, miR-126, miR-143, miR-145, miR-188, miR-200b, miR-219, miR-331
Breast	miR-17-5p, miR-21, miR-29b-2, miR-126, miR-143, miR-145, miR-146, miR-155, miR-181b-1, miR-188, miR-200b, miR-210, miR-213, miR-219 miR-331
Colon	miR-17-3p, miR-17-5p, miR-20a, miR-21, miR-24-1, miR-24-2, miR-29b-2, miR-30c, miR-32, miR-106a, miR-107, miR-126, miR-128b, miR-130a, miR-143, miR-145, miR-155, miR-188miR-191, miR-195, miR-200b, miR-218-2, miR-219, miR-221, miR-331, miR-223
Lung	miR-17-5p, miR-21, miR-30a, miR-126, miR-128b, miR-143, miR-145, miR-155, miR-188, miR-189, miR-191, miR-199a-1, miR-200b, miR-210,miR-213, miR-219, miR-223, miR-331
Pancreas	miR-17-5p, miR-20a, miR-21, miR-24-1, miR-24-2, miR-25, miR-29b-2, miR-30c, miR-32, miR-92-2, miR-106a, miR-107, miR-126, miR-128b, miR-143, miR-145, miR-146, miR-181b-1, miR-188, miR-191, miR-199a-1, miR-200b, miR-214, miR-218-2, miR-219, miR-221, miR-331
Prostate	miR-17-5p, miR-20a, miR-21, miR-25, miR-29b-2, miR-30c, miR-32, miR-92-2, miR-106a, miR-126, miR-143, miR-145, miR-146, miR-181b-1, miR-188, miR-191, miR-200b, miR-214, miR-218-2, miR-219, miR-223, miR-331
Stomach	miR-21, miR-24-1, miR-24-2, miR-25, miR-92-2, miR-107, miR-191, miR-199a-1, miR-214, miR-218-2, miR-221, miR-223
Thymus	miR-21, miR-126, miR-143, miR-145, miR-188, miR-200b, miR-219, miR-331

Stratagene's miRNA Workflow Overview



Sample QC

Sample Preparation	miRACLE miRNA Purification Kit ●Absolutely RNA SideStep ● miRNA 1st Strand cDNA Synthesis		
RNA	MVP Total Human RNA		
Labeling	High-Specificity miRNA QPCR Core Kit		
Detection	QRT-PCR	Specific miRNA primers	MX3000/3005P
Validation	QRT-PCR	Microarray	

miRNA products from Agilent Technologies

- Microarray Platform:
  -Human miRNA microarray kit (version 1.0)
  -miRNA labeling reagent and hybridization kit
  -March 1: Mouse, Rat and Human (version 2.0) microarray kits
- 2100 Bioanalyzer:
  Total RNA Assays (for RNA integrity)
  -RNA 6000 Nano Kit
  -RNA 6000 Pico Kit
  -Small RNA Kit (for analysis of small RNAs)

- Stratagene's qPCR:
  -High-Specificity miRNA QRT-PCR Detection Kit
  -miRNA Specific Forward Primers