miRNAs are promising novel biomarkers

Inserm/University Paul Sabtier

NOvember 22, 2011

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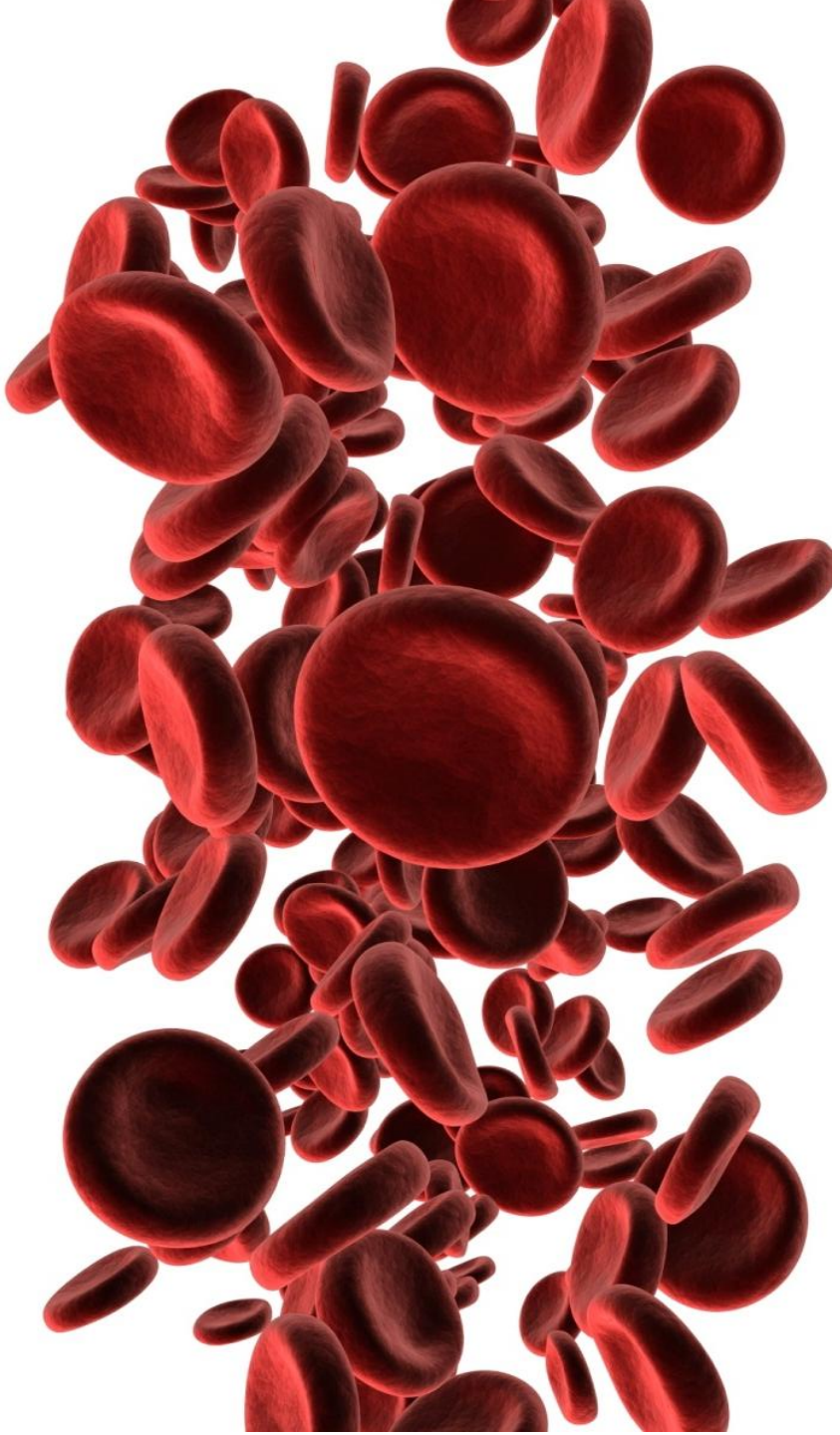

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Exiqon at a glance

Highlights

- Proprietary LNA™ detection technology
- Unique IP to novel group of miRNA biomarkers
- Established one-stop shop for Life Science products
- Unique platform for Molecular Diagnostics products
- Multiple license agreements with world leading companies
- Listed on NASDAQ OMX, Copenhagen (”EXQ”)
- 75 employees
- Founded 1996

Locations

Exiqon at a glance

Business divisions

EXIQON Life Sciences

Exiqon Life Sciences combines leading-edge scientific expertise in gene expression with our proprietary LNA™ technology. Our products, services and scientific staff enable life science researchers to make groundbreaking discoveries

EXIQON Diagnostics

Exiqon Diagnostics is the leader in providing technologies for miRNA biomarker detection. Exiqon Diagnostics is dedicated *in collaboration with partners* to develop novel molecular diagnostic tests for early detection of diseases and knowledge based treatment selection.

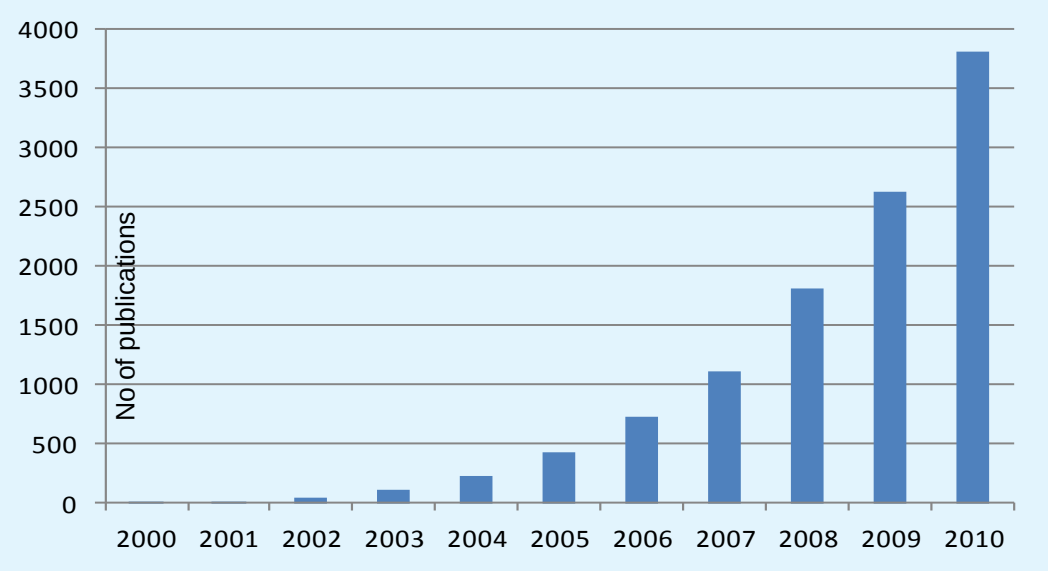
MicroRNA research: Broad product portfolio

Process	Isolation	Expression Analysis		Localisation	Functional Analysis
Product	 miRCURY™ Sample Isolation System	 miRCURY LNA™ miRNA Array System	 miRCURY LNA™ miRNA PCR System	 miRCURY LNA™ miRNA Detection Probes	 miRCURY LNA™ miRNA Inhibitor System
Launch	2009	2006; 6th gen 2010	2nd gen. 2009	2005 & 2009	2006 & 2009

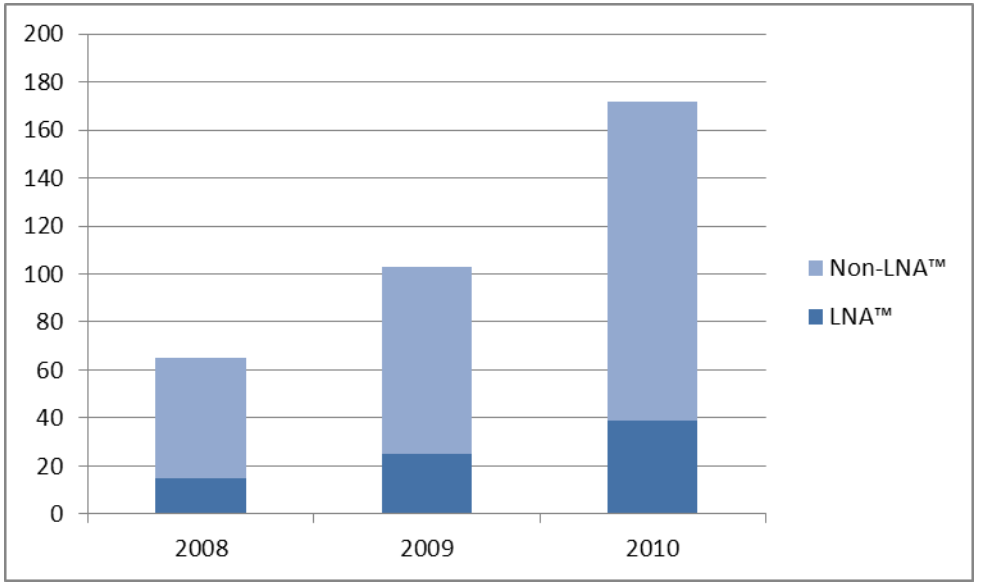
Also available as services

Number of miRNA publications strongly growing

Number of miRNA publications 2000-2010



Number of miRNA publications Oct 2008 / Oct 2009 / Oct2010



- How to study microRNAs – introduction to LNA™
- miRCURY LNA™ microRNA arrays
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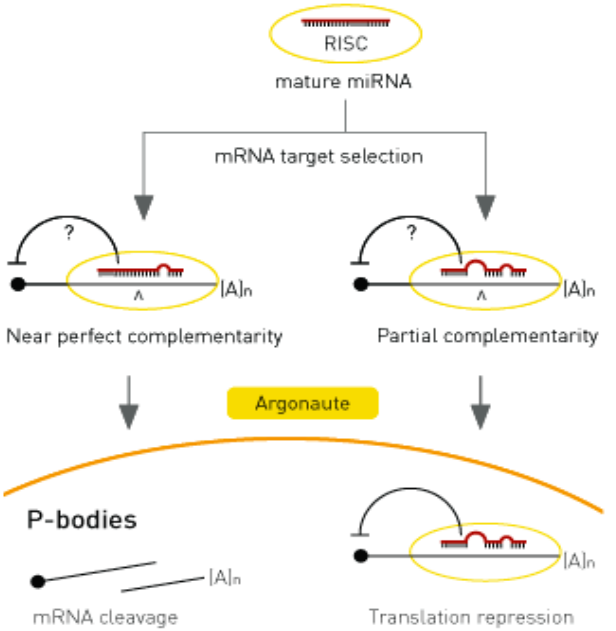
Six facts about microRNAs

Six Facts

1. Short non-coding RNA molecules of 19-22 nucleotides
2. Post-transcriptional regulators of mRNA
3. 1424 annotated human microRNAs* (1000 - 2000 predicted)
4. Regulate at least one third of all human genes
5. Phylogenetically well conserved
6. Altered microRNA expression profiles are associated with a number of diseases (cancer, diabetes, neurological disease, viral infection)

*Sanger miRBase release 17.0, April 2011
http://www.mirbase.org

microRNA Mode of Action



Binding to 3' UTR of mRNA

- Imperfect complementarity **translational repression mRNA decay**
- Perfect complementarity **mRNA cleavage**

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Reasons to use miRNAs as biomarkers

Key roles in pathway regulation & tissue differentiation

•Important regulatory roles in many diseases

•Regulate expression of key proteins in drug response pathways

•Predict response to targeted therapies

•**Actively secreted into the circulation**

•Relatively small number of genes to profile

•Huge dynamic range (0 to 40,000 molecules per cell)

Highly stable in clinical sample preparations

- archival FFPE material
- **serum and plasma (routinely collected, minimally invasive)**
- other body fluids

Analyzing miRNAs – challenges using traditional DNA technology

Feature	Challenge
Very short sequences ~ 22nt The length of a normal capture probe or PCR primer	Hard to achieve sufficient sensitivity - especially with AT-rich sequences. **No design possible!**
There are many microRNA families, members differ by single nucleotides	Hard to achieve sufficient specificity to discriminate between family members
Highly variable GC content – from 5-95%	A serious problem with multiplex assays (such as microarrays – full length DNA probes have a Tm span of 45 C!)

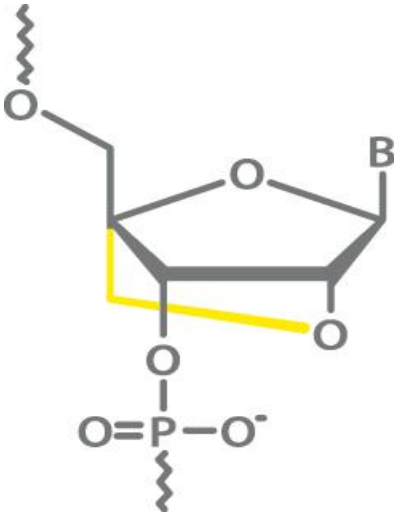
LNA™ technology overview

Key advantages

Technology allows for improved binding

- LNA is a bicyclic high affinity RNA mimic with the sugar ring locked in the 3’-endo conformation
- Stable A-helix with good base-stacking
- Increased Tm (Tm increases by 2 - 8ºC per base)
- Improved mismatch discrimination
- High sensitivity and specificity in hybridization assays
- Obeys Watson-Crick base-pairing rules
- Easy to implement in standard oligo synthesis

LNA™



K. Bondensgaard *et al.*, *Chem. Eur. J.* **2000**, *6*, 2687
M. Petersen *et al.*, *J. Am. Chem. Soc.* **2002**, *124*, 5974

LNA™ technology enables design of short RNA analysis tools

The power of LNA™ to modulate affinity of probes for RNA target[1]

DNA oligo	caacatcagtctgataagct	47 C
LNA/DNA mixmer (1 LNA)	caacatcag**T**ctgataagct	52 C
LNA/DNA mixmer (2 LNAs)	caacatcag**T**c**T**gataagct	62 C
LNA/DNA mixmer (4 LNAs)	caaca**T**cag**T**c**T**ga**T**aagct	73 C
LNA/DNA mixmer (6 LNAs)	caa**C**a**T**cag**T**c**T**ga**T**a**A**gct	78 C
LNA/DNA mixmer (8 LNAs)	c**A**a**C**a**T**cag**T**c**T**ga**T**a**A**g**C**t	84 C

Discriminative power of LNA™ [2]

Target / Probe	Perfect match DNA	Single mismatch 3'ccaggaa**g**gaaccac-5'	ΔT_m
DNA 15mer 5'ggtcctt**a**cttggtg3'	T_m = 59,4 C	T_m = 55,7 C	3,7 C
DNA/LNA 15mer 5'ggtcctt**A**cttggtg3'	T_m = 60,9 C	T_m = 52,7 C	8,2 C

- **LNA enhances affinity of oligonucleotides for complementary RNA and DNA targets**
- **LNA™ technology enables probe design with a desired Tm for short RNA targets**

- **LNA enhances specificity of oligonucleotides for complementary RNA and DNA targets**

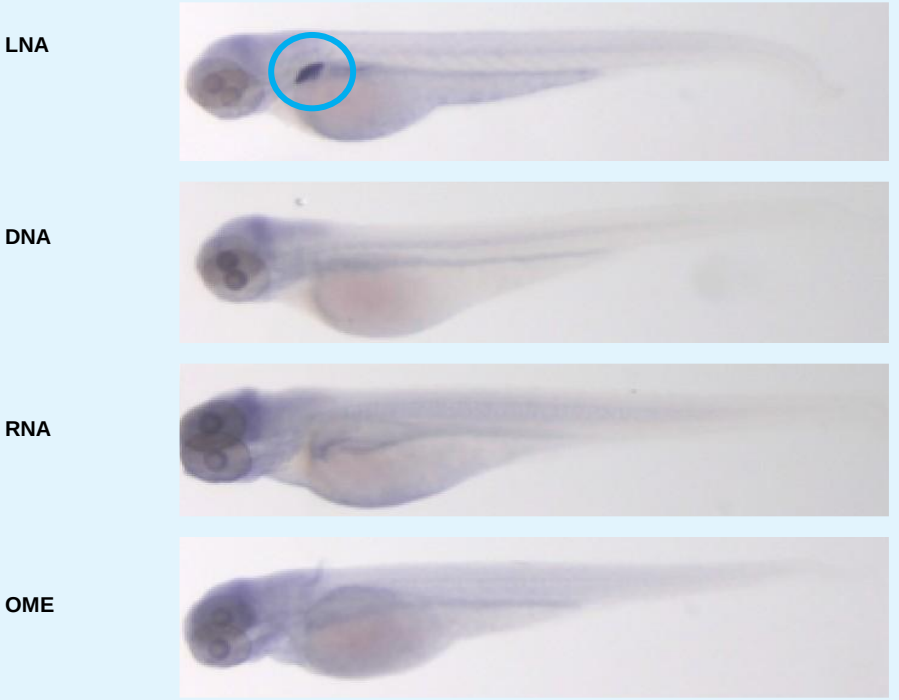
[1] Capital letters in red indicate LNA™

[2] ΔTm is the difference between *T*m of perfect match and mis-match duplexes. Capital letters indicate LNA™

Sensitivity and specificity of LNA™ (Locked Nucleic Acid) technology

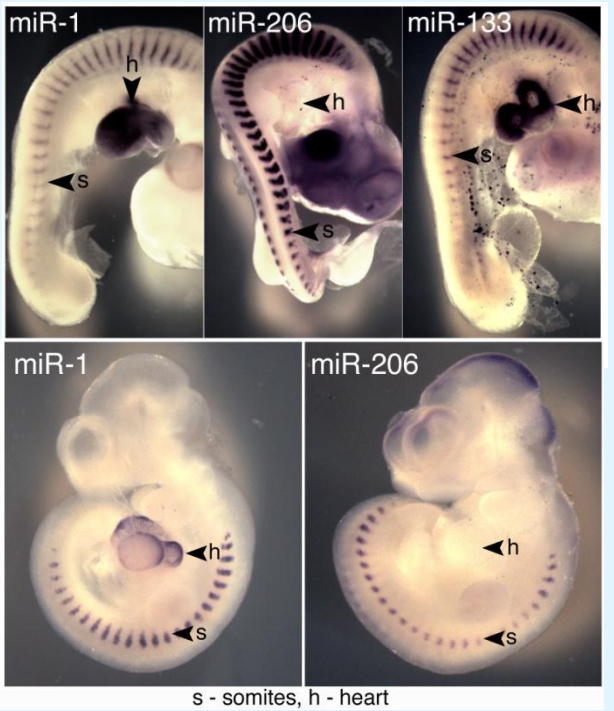
Sensitivity achieved with LNA

LNA™ vs. competing technology



In situ hybridizations using DIG-labeled probes against liver specific and highly expressed miR-122.

Specificity achieved with LNA



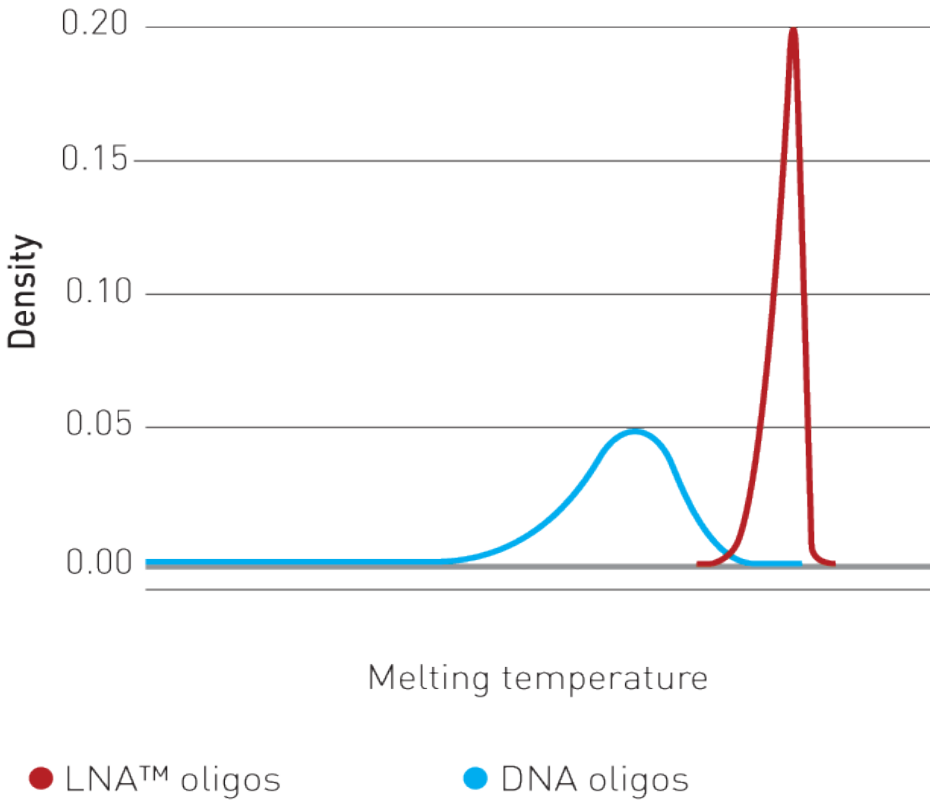
s - somites, h - heart

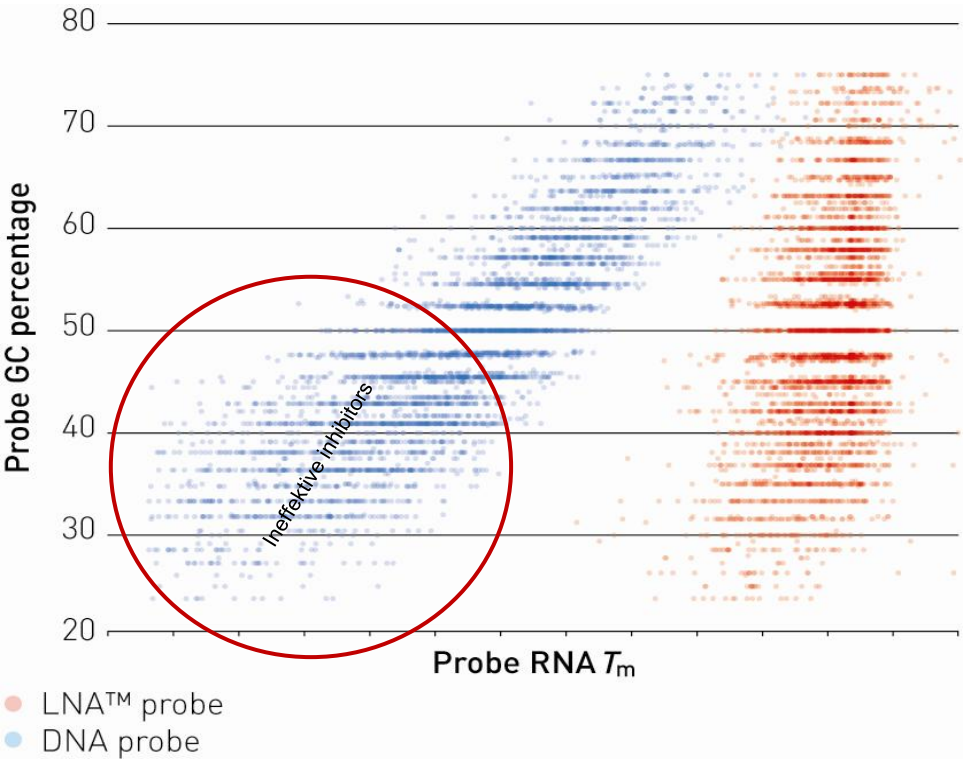
Pictures kindly provided by D. Sweetman, Münsterberg Group, University of East Anglia, Norwich, UK

miR-206 UGGAAUGUAAGGAAGUGUGUGG[12]
miR-1 UGGAAUGUAAAGAAGUAUGUAU

T_m normalization of microRNA inhibitors achieved by LNA design

Tm normalization ensures high potency of LNA inhibitors regardless of target microRNA GC content





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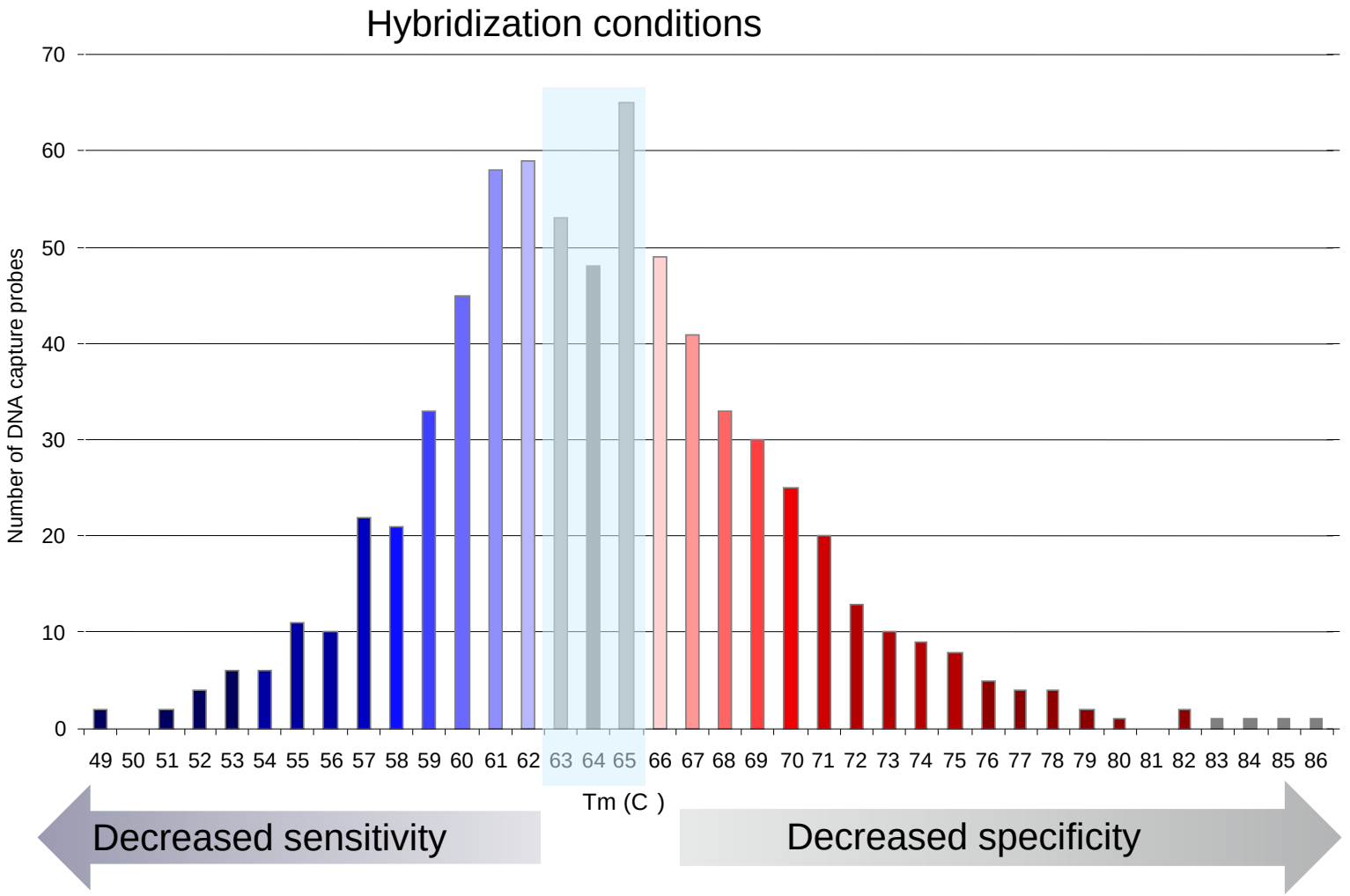
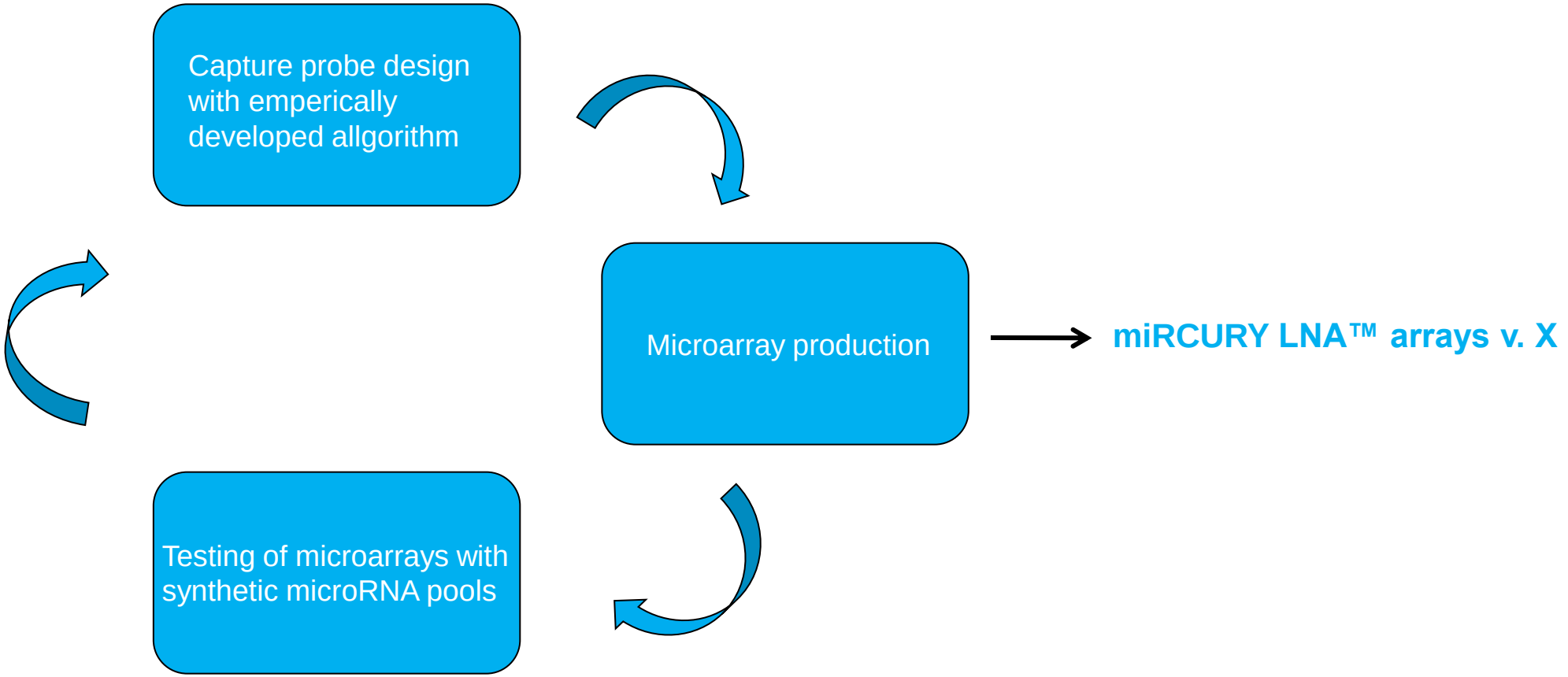
DNA capture probes have poor sensitivity for low Tm targets and decreased specificity for high Tm targets



Development of miRCURY LNA™ microRNA array platform

Capture probes are experimentally validated



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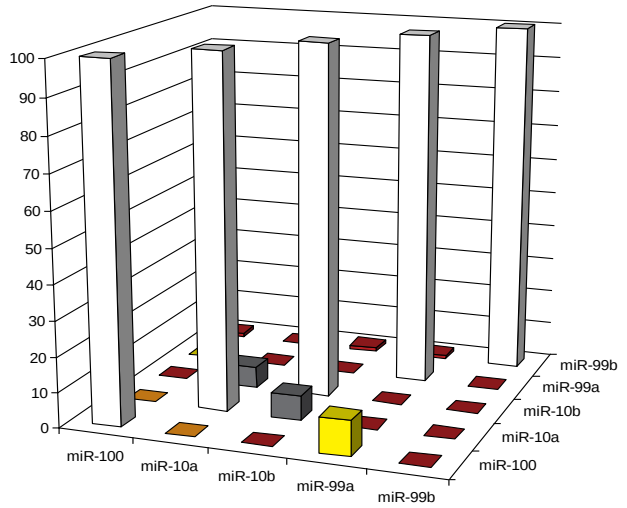
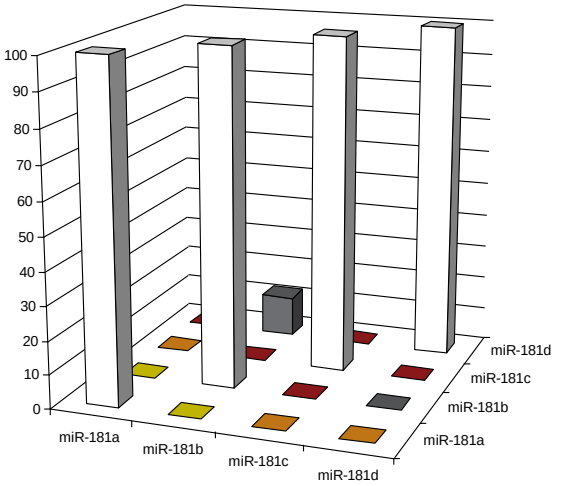
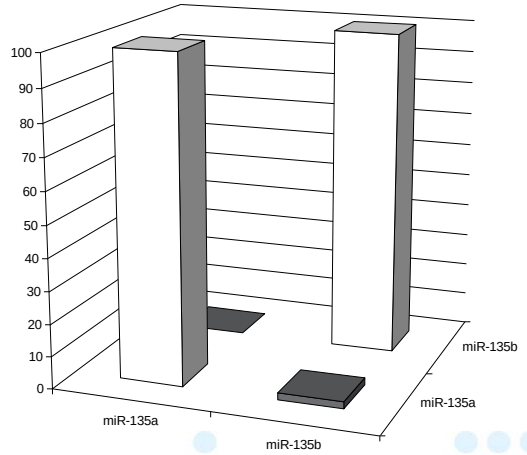
Testing specificity of capture probes with synthetic miRNA pools

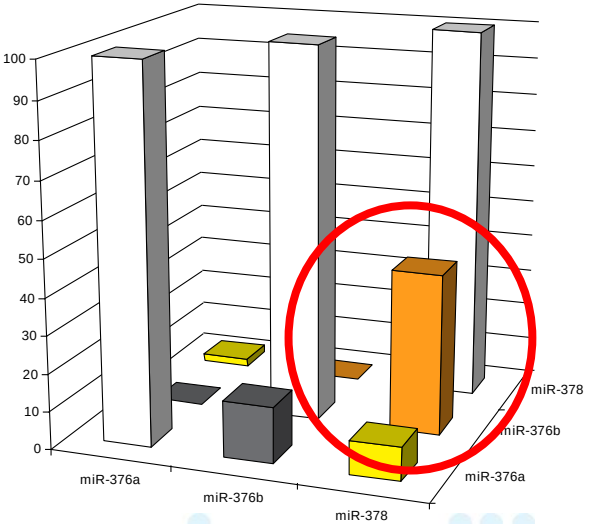


Number of mismatches

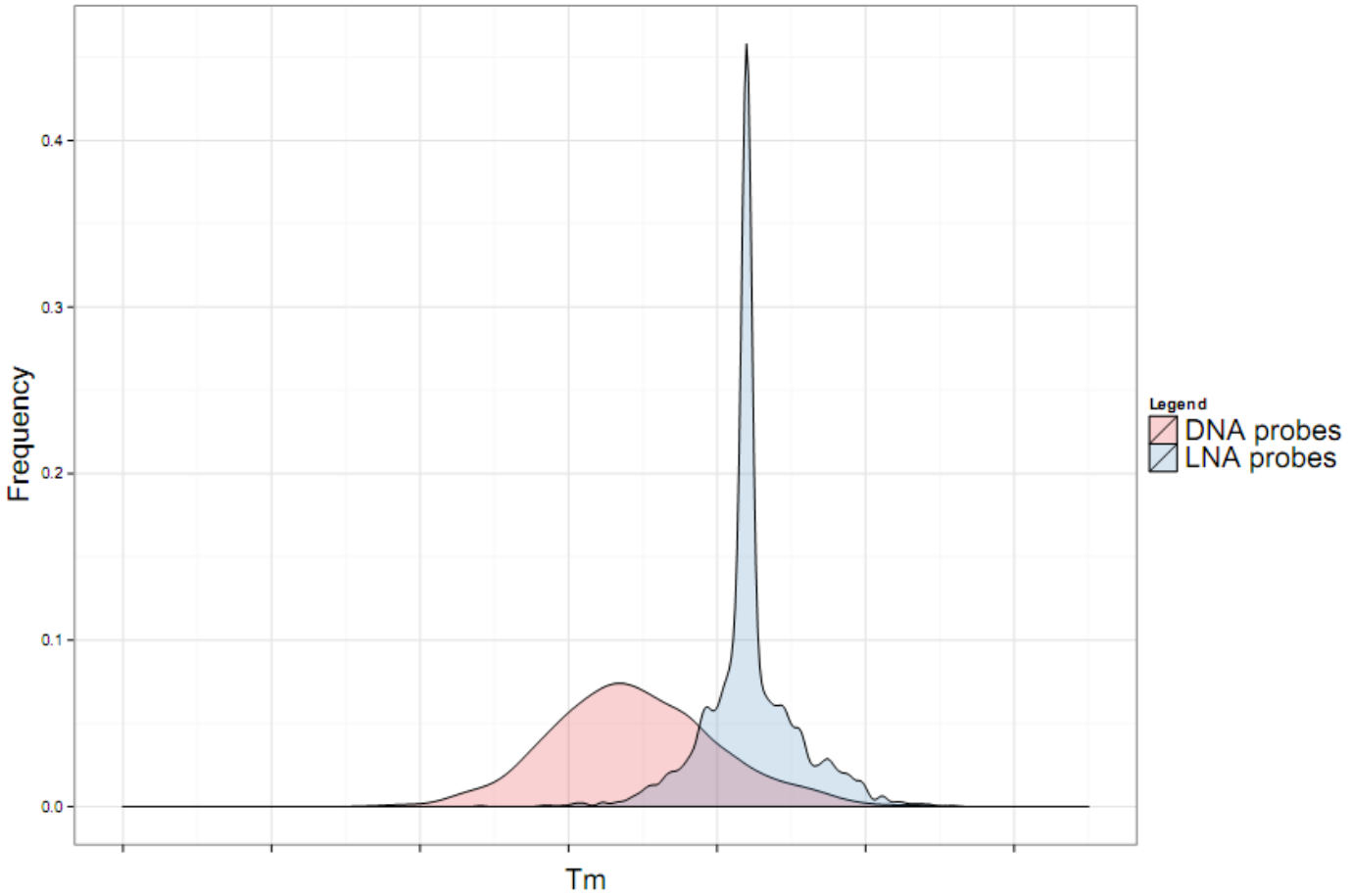
0
1
2
3
>4





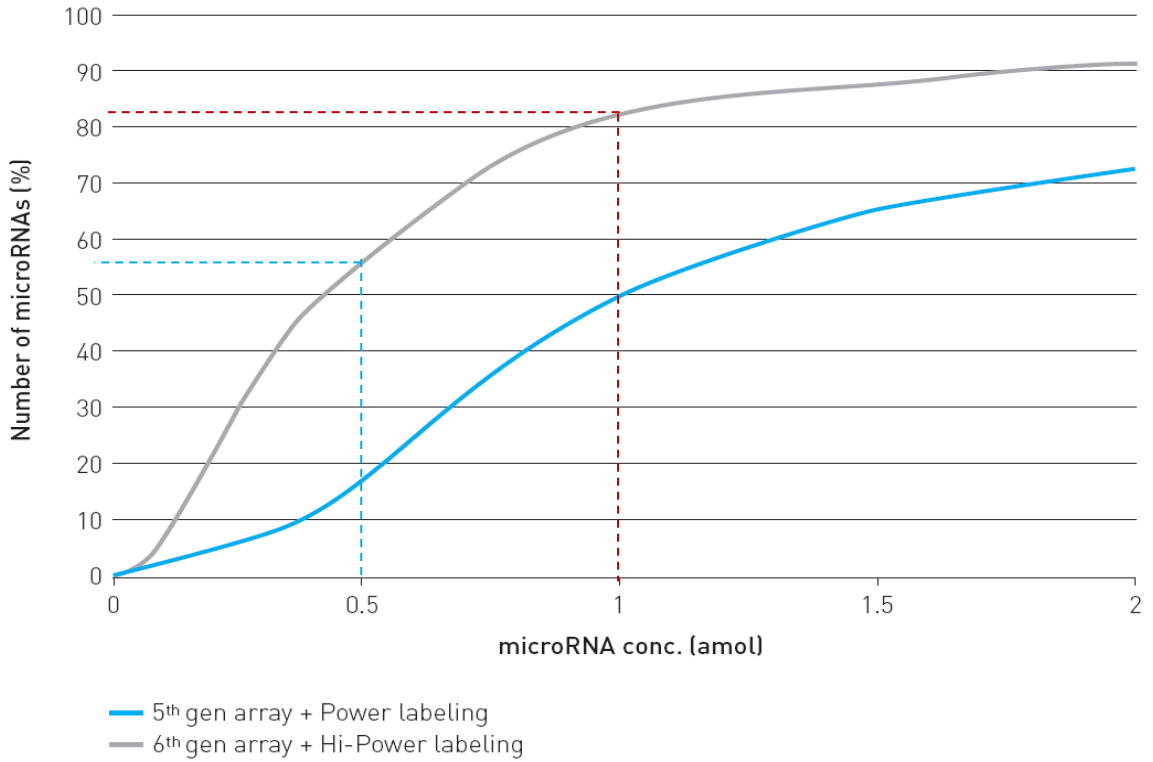


miRCURY LNA™ microRNA arrays have Tm normalized capture probes



Using LNA™, the Tm is increased significantly and the Tm range is narrowed significantly, compared to DNA probes. This results in increased stringency and optimal hybridization conditions for the LNA™ capture probes.

Sensitivity of 6th generation miRCURY LNA™ microRNA arrays

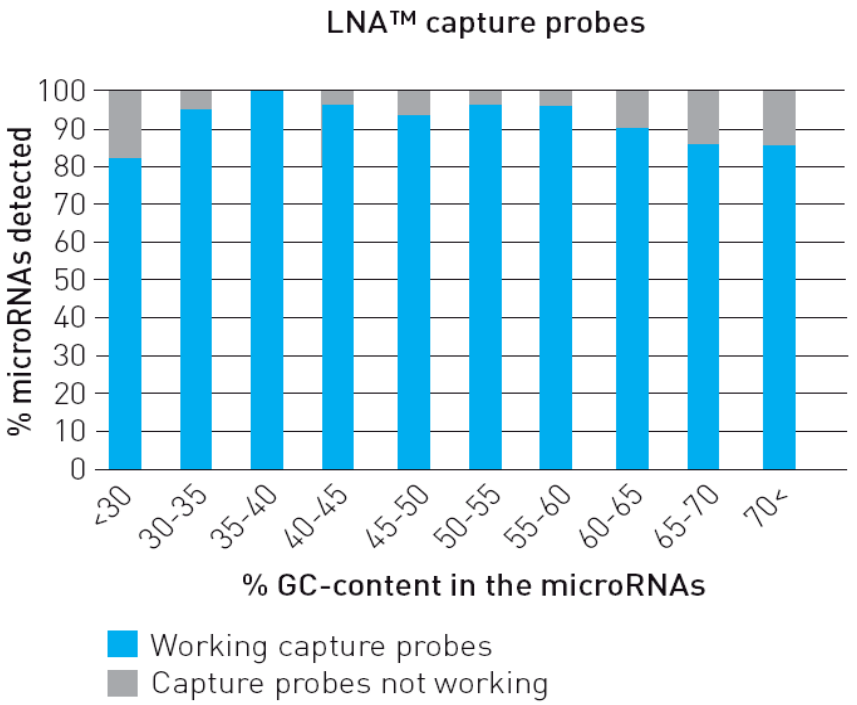


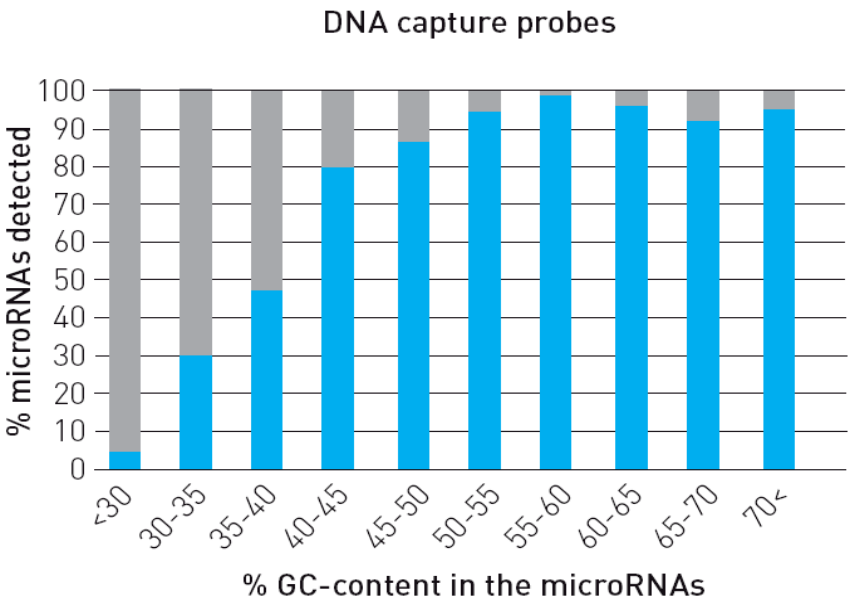
>50% of all microRNA are detected at 0.5 amol

82% of all microRNAs are detected at 1 amol

0.5 amol ~ 5 copies/cell, using 1 µg total RNA

miRCURY LNA™ microRNA Arrays have unmatched sensitivity for ALL microRNAs – even the AT-rich





DNA-based arrays fail to detect low GC-content microRNAs **(>20% of all microRNAs)**

Excellent discrimination between closely related microRNAs – let-7 family specificity

hsa-let-7 family sequences

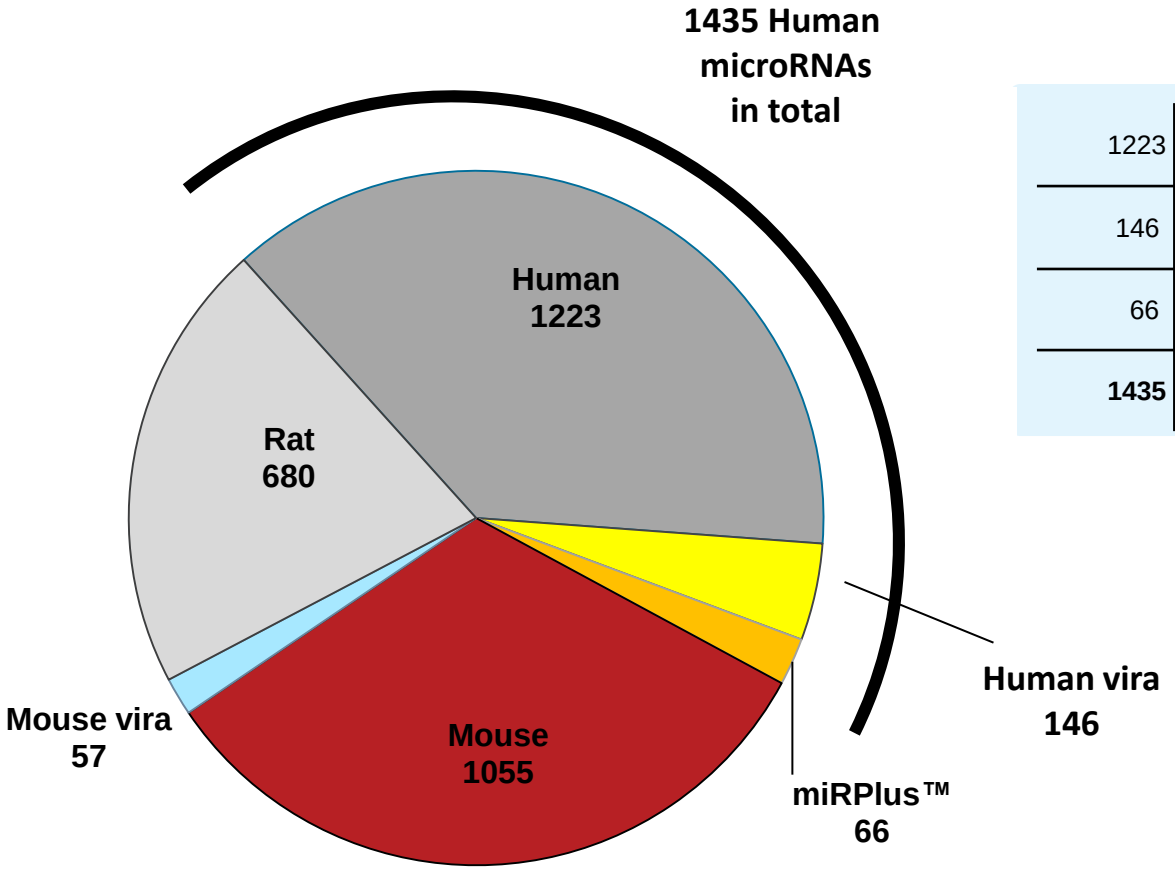
```
hsa let-7a    UGAGGUAGUAGGUUGUAUAGUU
hsa let-7b    UGAGGUAGUAGGUUGUGUGGUU
hsa let-7c    UGAGGUAGUAGGUUGUAUGGUU
hsa let-7d    AGAGGUAGUAGGUUGCAUAGU-
hsa let-7e    UGAGGUAGGAGGUUGUAUAGU-
hsa let-7f    UGAGGUAGUAGAUUGUAUAGUU
hsa let-7g    UGAGGUAGUAGUUUGUACAGU-
hsa let-7i    UGAGGUAGUAGUUUGUGCUGU-
```

microRNA spike-in

Array capture probes

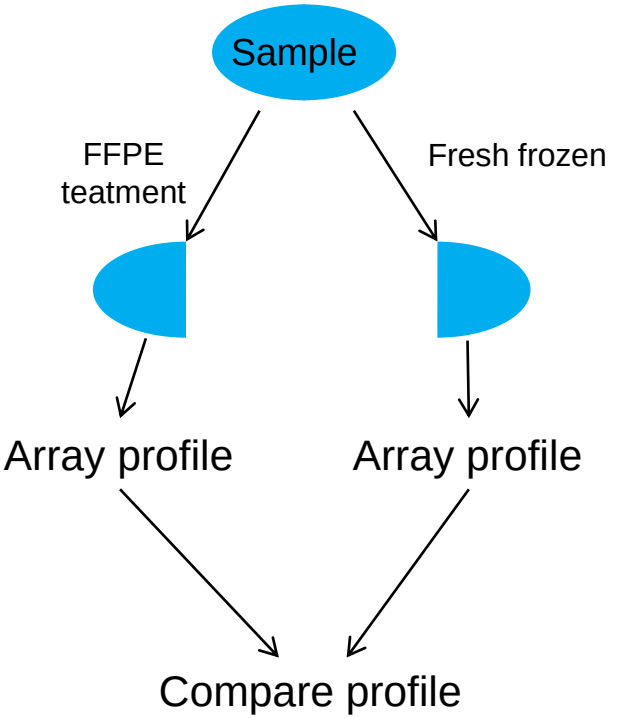
	let-7a	let-7b	let-7c	let-7d	let-7e	let-7f	let-7g	let-7i
let-7a	100	2	17	4	4	2	1	2
let-7b	1	100	4	1	1	1	1	1
let-7c	0	8	100	0	1	0	0	0
let-7d	2	2	5	100	1	0	0	0
let-7e	1	0	0	0	100	0	0	0
let-7f	6	3	5	3	3	100	2	3
let-7g	0	0	1	0	0	1	100	4
let-7i	0	3	0	0	0	0	2	100

Coverage – miRBase v. 16.0



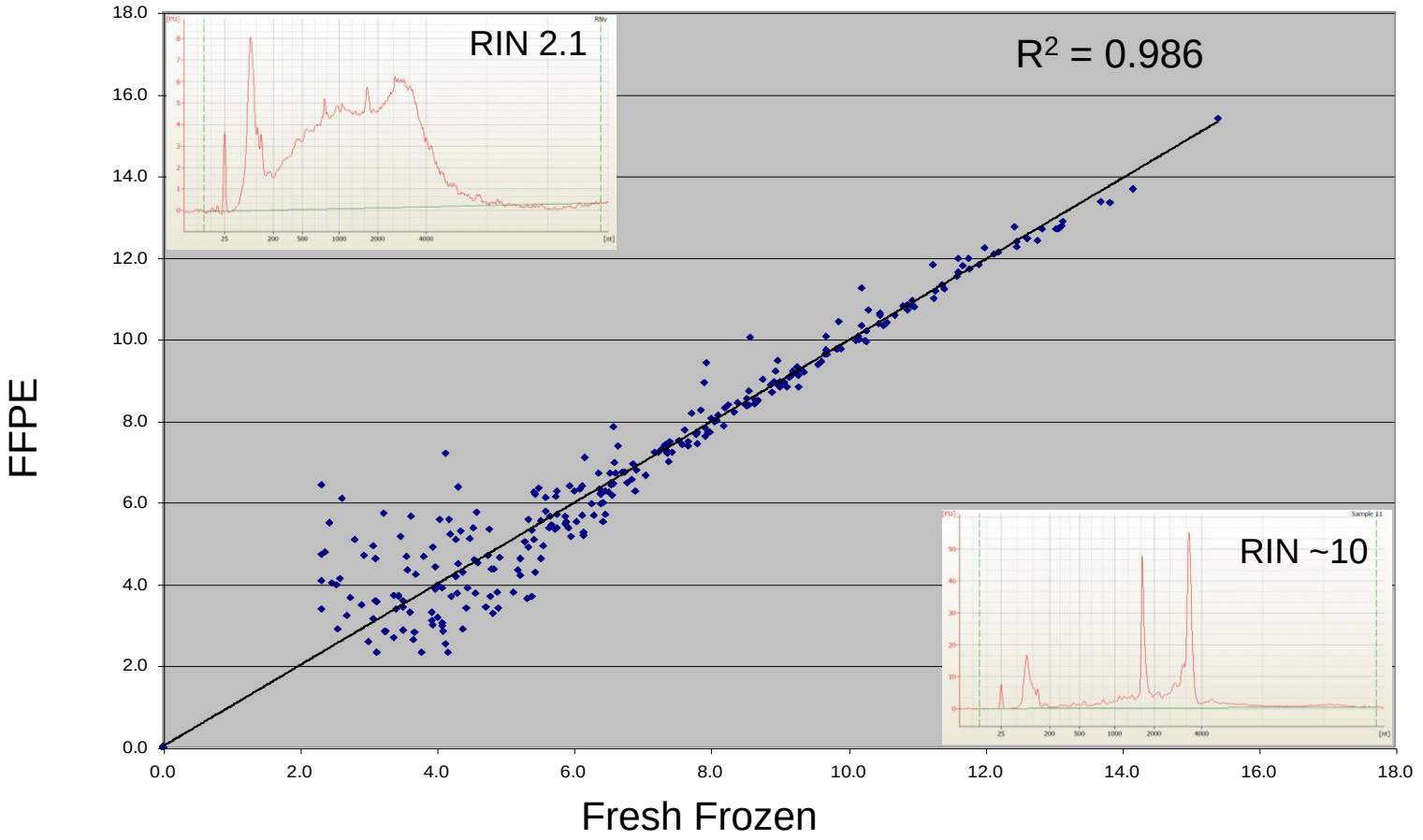
1223	human microRNAs (miRBase 16)
146	human viral microRNAs (miRBase 16)
66	miRPlus™ human microRNA candidates
1435	**Human microRNAs in total**

FFPE samples –
testing the performance of the miRCURY LNA™ microRNA Array



microRNAs from FFPE vs. fresh frozen tissue

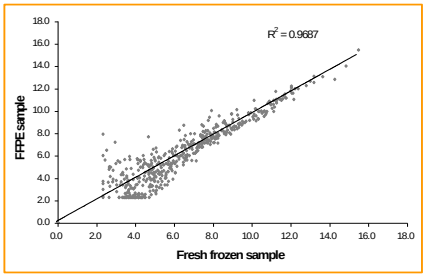
Kidney:



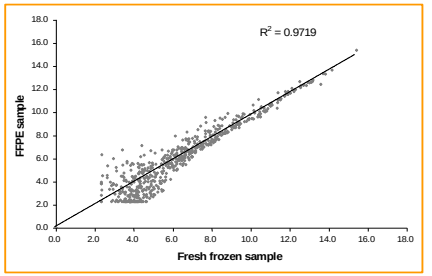
24

Excellent correlation of microRNA profiles from FFPE and fresh frozen samples

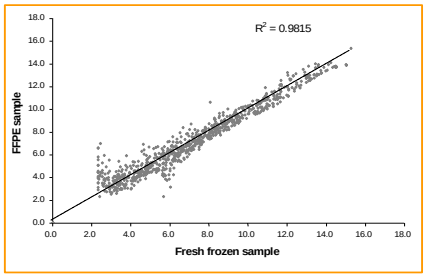
Liver



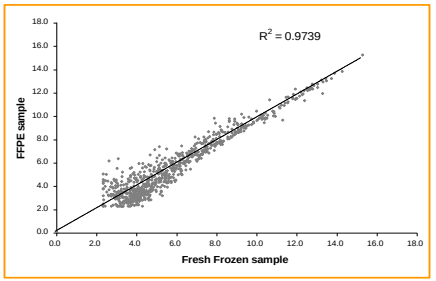
Kidney



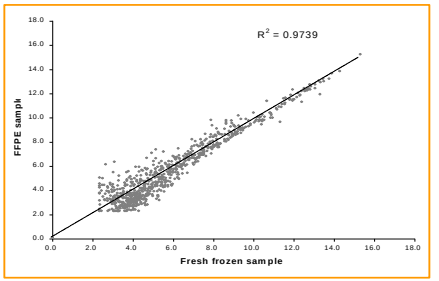
Jejunum



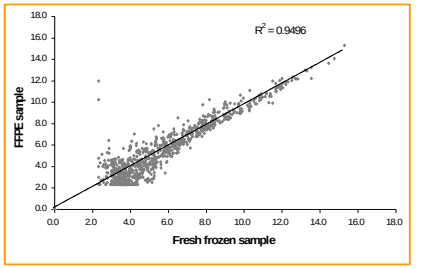
Heart



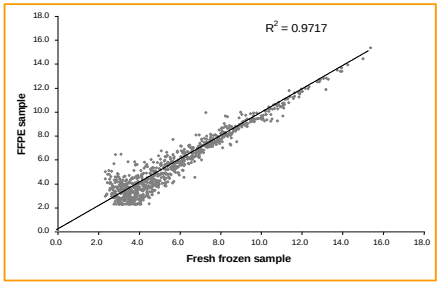
Pancreas



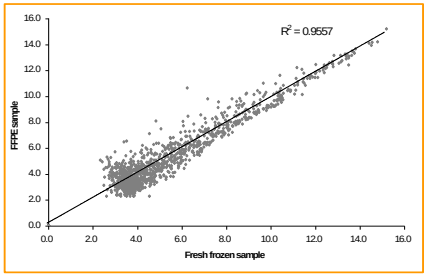
Muscle



Brain

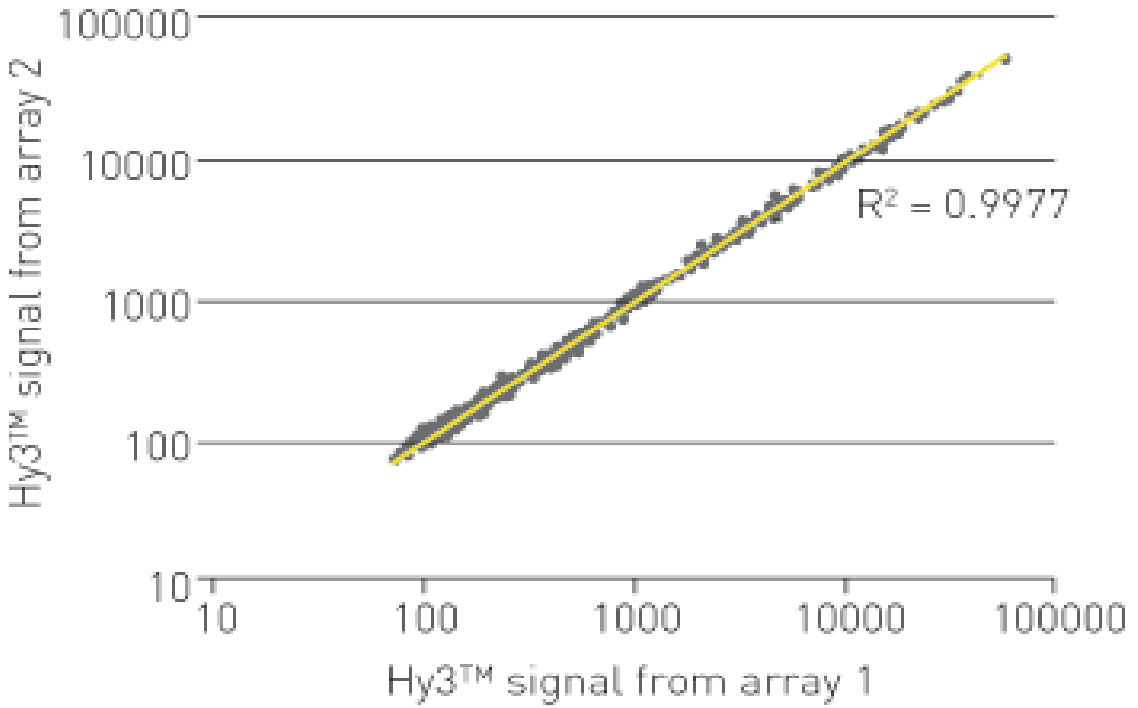


Lung



LNA-based arrays makes profiling of microRNA from FFPE samples possible

Minimal batch to batch variation – ideal for single color expression profiling



Very low CV between different batches of the array:

median CV ≤ 10%

median CV 2-4% typically obtained between different batches

enabling superior
single color experiments

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Identification and validation of diagnostic microRNA biomarkers for skin cancer (Cutaneous T-cell lymphoma)

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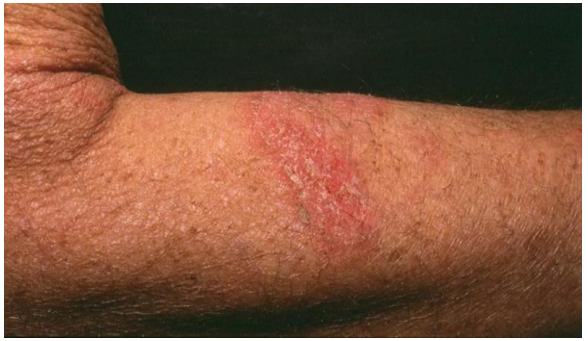
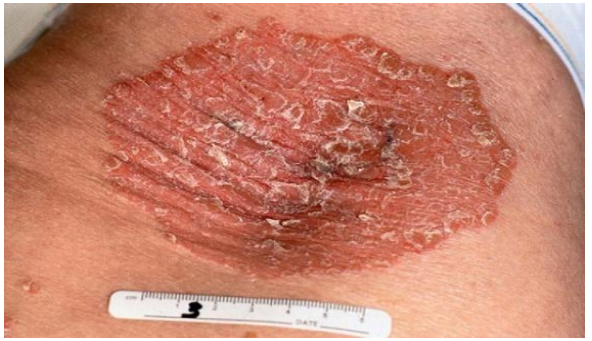
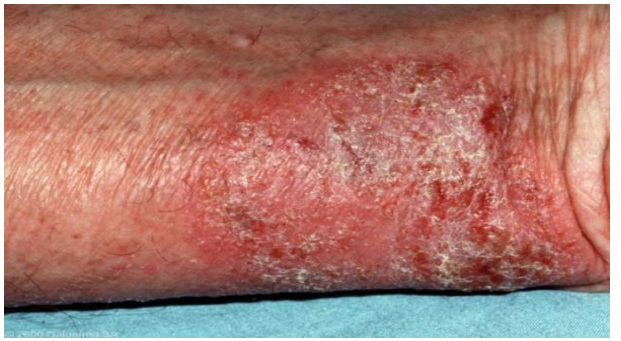
- CTCL are the most frequent primary lymphomas of the skin
- Early diagnosis of CTCL has important consequences for therapeutic options and determination of prognosis
- Early diagnosis of CTCL is difficult due to the great clinical, pathological, and histological resemblance to benign inflammatory skin diseases (psoriasis and eczema).
- From initial skin lesion to definite CTCL diagnosis: 6 years (median)



Cutaneous T-cell lymphoma (CTCL)



Psoriasis



Eczema

* Farber EM, Nall L. Epidemiology: Natural History and Genetics. In: Roenigk HH Jr, et al, editors. Psoriasis, 3rd edn. Marcel Dekker, New York, 1998:107-157.

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Sample selection and experimental set up

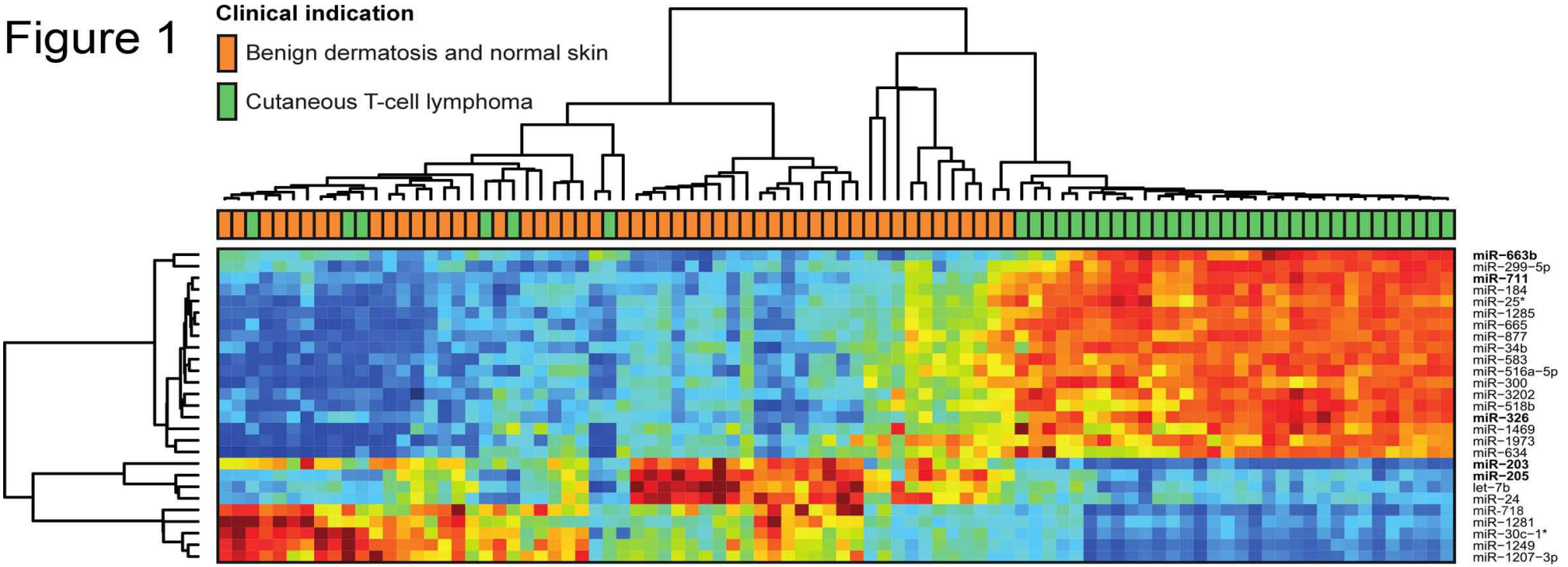
	Cutaneous Lymphoma (*n*=63)					Benign skin disease or normal skin (*n*=85)						*P*-value
	MF	SS	CALCL	NOS	Σ	AD	ND	PP	PN	NN	Σ	
Age (years) *												**<0.001**
<30	0	0	1	0	**1**	19	0	4	4	2	**29**	
30-44	5	0	0	0	**5**	1	0	6	3	0	**10**	
45-59	9	1	1	2	**13**	0	1	19	7	0	**27**	
60-74	14	6	2	2	**24**	0	3	12	2	0	**17**	
≥75	10	0	2	5	**17**	0	0	1	1	0	**2**	
Gender *												**1.00**
Male	23	7	5	5	**40**	8	1	30	16	2	**57**	
Female	15	0	1	4	**20**	12	3	12	1	0	**28**	
Microarray batch												**1.00**
1	8	2	1	2	**13**	4	1	8	3	1	**17**	
2	7	2	2	1	**12**	4	1	8	3	1	**17**	
3	7	1	2	2	**12**	4	1	8	3	0	**16**	
4	9	1	2	2	**14**	4	0	9	4	0	**17**	
5	8	1	1	2	**12**	4	1	9	4	0	**18**	

- For each sample RNA was extracted from 6 x 10µM FFPE sections (archival).
- Samples were divided into a training set of 90 and a test set of 58 (randomized) samples.
- 100ng total RNA was used per array.

Reference: Ralfkiaer et al, 2011. Blood
doi:10.1182/blood-2011-06-358382

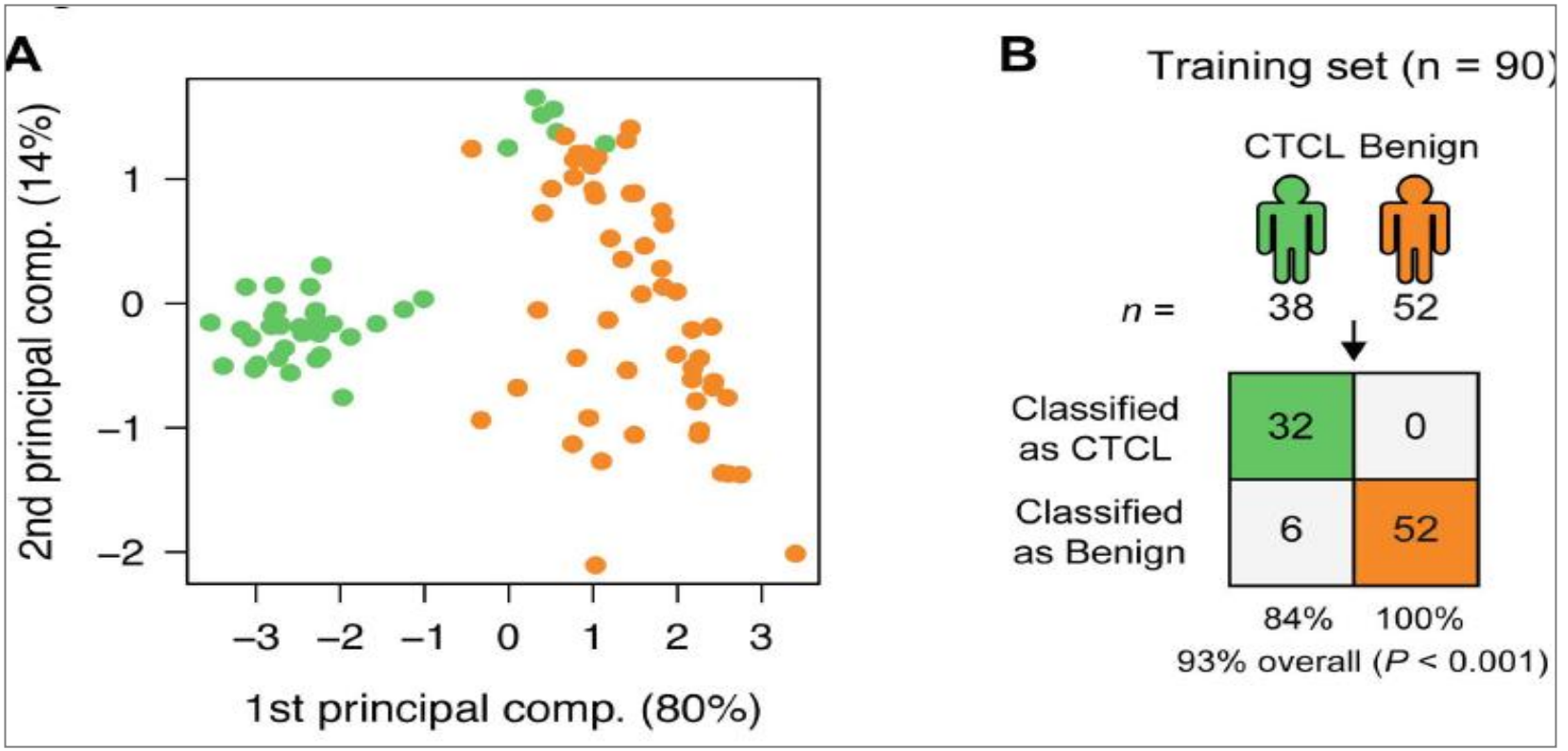
LNA™ microRNA arrays identify 27 highly significant microRNAs differentially expressed between CTCL and benign skin disease

Figure 1



Reference: Ralfkiaer et al, 2011. Blood
doi:10.1182/blood-2011-06-358382

Identification of a microRNA classifier for CTCL versus benign skin disease

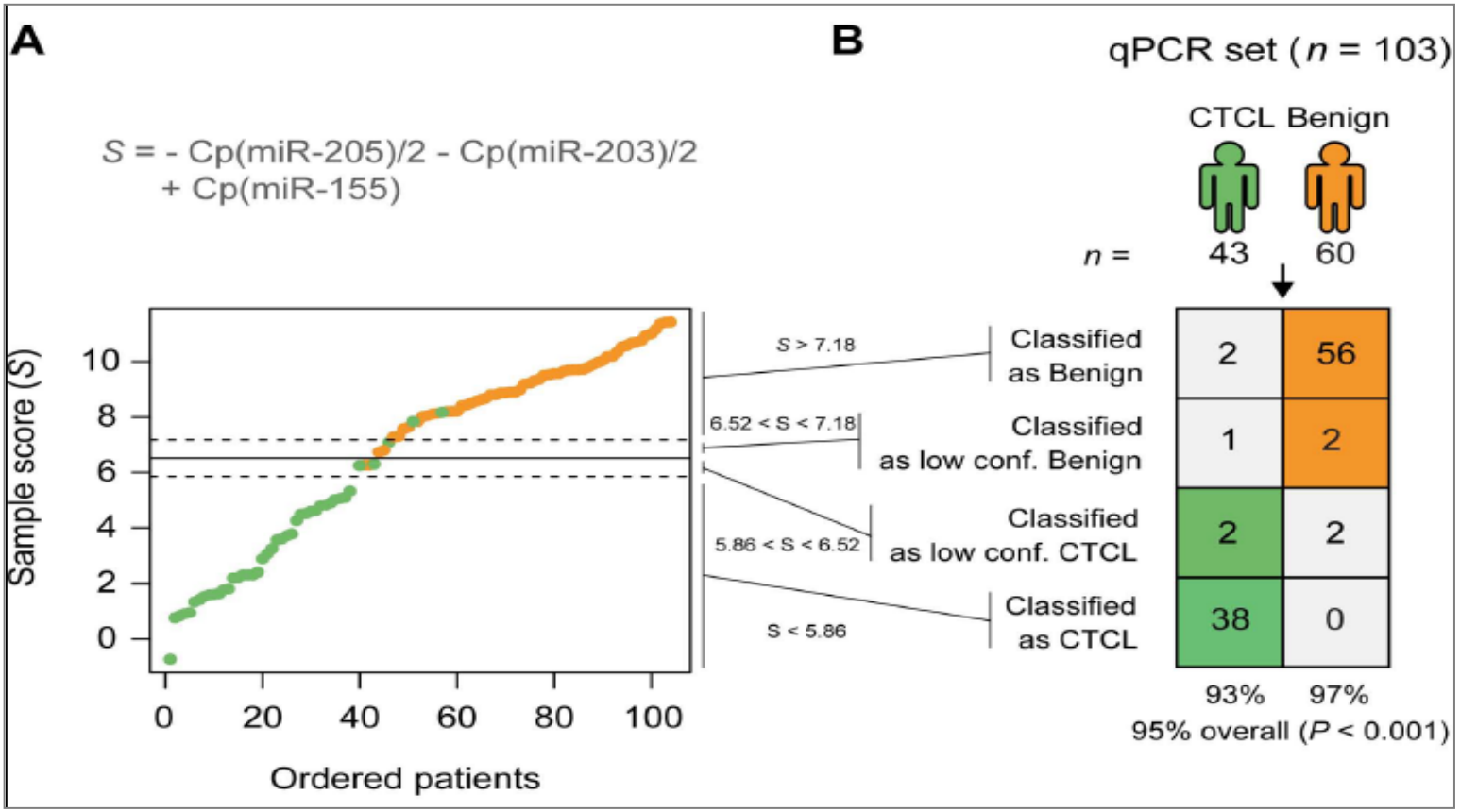


Reference: Ralfkiaer et al, 2011. Blood doi:10.1182/blood-2011-06-358382

The 5 most differentially expressed microRNAs from the microarray data make up the classifier: miR-203, miR-205, miR-326, miR-663b and miR-711.

The classifier is validated in the test sample set and in two independent sample sets including a mouse xenograph model.

Validation of a highly sensitive and specific microRNA classifier



miR-155 functions as an oncogene in other hematological malignancies

miR-203 is important in keratinocyte development and may act as a tumor suppressor

miR-205 is a tumor suppressor possibly through targeting VEGF

Reference: Ralfkiaer et al, 2011. Blood
doi:10.1182/blood-2011-06-358382

blood

Prepublished online August 24, 2011;
doi:10.1182/blood-2011-06-358382

Diagnostic microRNA profiling in cutaneous T-cell lymphoma (CTCL)

Ulrik Ralfkiaer, Peter H. Hagedorn, Nannie Bangsgaard, Marianne B. Løvendorf, Charlotte B. Ahler, Lars Svensson, Katharina L. Kopp, Marie T. Vennegaard, Britt Lauenborg, John R. Zibert, Thorbjørn Krejsgaard, Charlotte M. Bonefeld, Rolf Søkilde, Lise M. Gjerdrum, Tord Labuda, Anne-Merete Mathiesen, Kirsten Grønbaek, Mariusz A. Wasik, Malgorzata Sokolowska-Wojdylo, Catherine Queille-Roussel, Robert Gniadecki, Elisabeth Ralfkiaer, Carsten Geisler, Thomas Litman, Anders Woetmann, Christian Glue, Mads A. Røpke, Lone Skov and Niels Odum

- How to study microRNAs – introduction to LNA™
- miRCURY LNA™ microRNA arrays
- Early diagnosis of cutaneous T-cell lyphoma
- Detection of microRNAs in clinical samples and diagnostic development using LNA™ Universal RT microRNA PCR
- Early detection of colorectal cancer from human patient blood plasma

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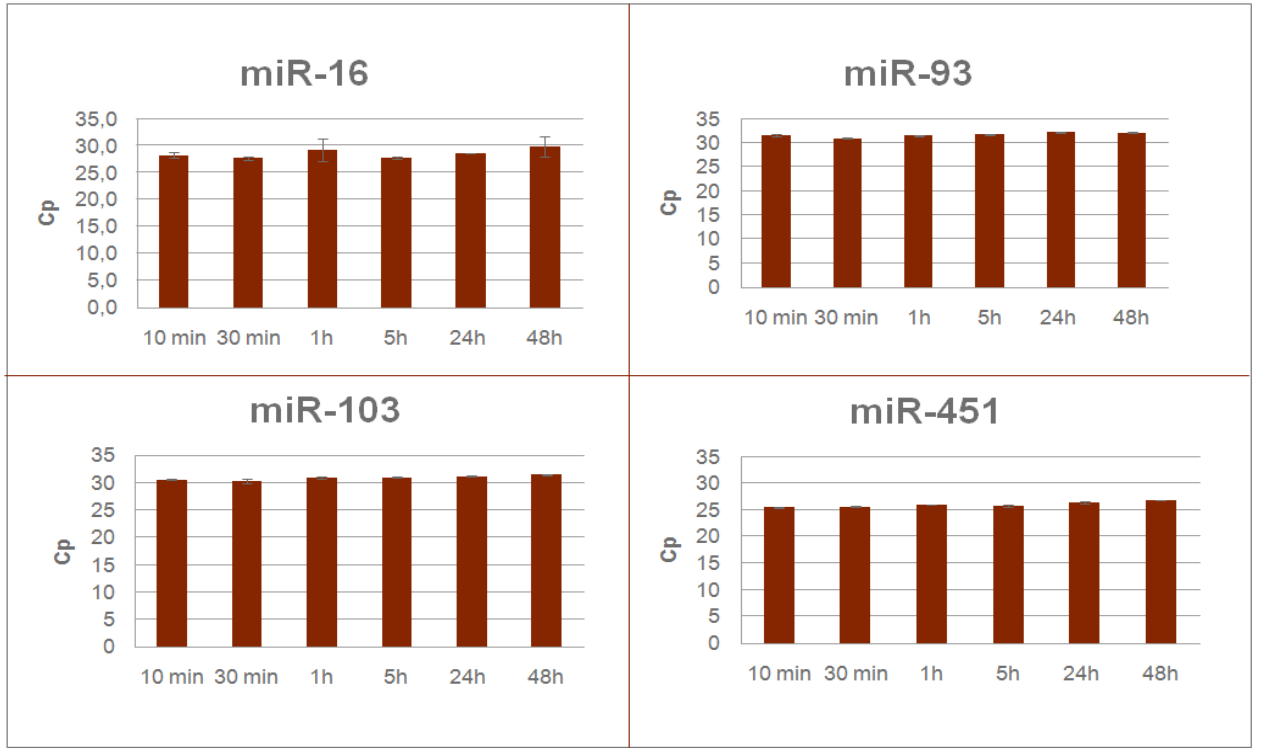
Circulating microRNAs in cell-free serum and plasma
- Major potential as biomarkers

lung, **colon cancer** and **diabetes** have specific serum-miRNA profiles	Chen *et al.,* 2008, Cell Res, 1-10
MiR-92 is significantly elevated in plasma of patients with CRC, potential **non-invasive molecular marker for CRC screening**	Ng *et al.*, 2009, Gut, **58:**1375-1381
Serum miR-21 associated with relapse-free survival in diffuse large cell B **lymphoma**	Lawrie *et al.,* BJH, 2008
human prostate cell line xenotransplanted into mice and tumor-derived human specific miRNAs identified in plasma	Mitchell *et al.,* 2008, PNAS, 105(30):10513.
microRNAs differentially expressed in serum of **ovarian cancer** patients	Resnick *et al.,* 2009 Gyn Oncology 112: 55-59
microRNAs differentially expressed in circulating tumor-derived **exosomes** of **ovarian cancer** patients	Taylor *et al.,* 2008, Gyn Oncology, 110:13
The circulating microRNA-221 level in patients with malignant melanoma as a new tumor marker	Kanemaru H *et al*., 2011, J Dermatol Sci. 61(3):187-93
Plasma miR-208 as a Biomarker of **Myocardial Injury**	Ji *et al.,* 2009, Clin. Chem. 55: 11
Plasma miRNA signature for diabetes mellitus	Zampetaki *et al.,* 2010, Circ Res. 2010;107:810-817

Clinical QC of miRNA stability in blood derived Plasma

UNIVERSITY OF COPENHAGEN FACULTY OF LIFE SCIENCES

Stability of miRNAs in plasma stored at room temperature before extraction.

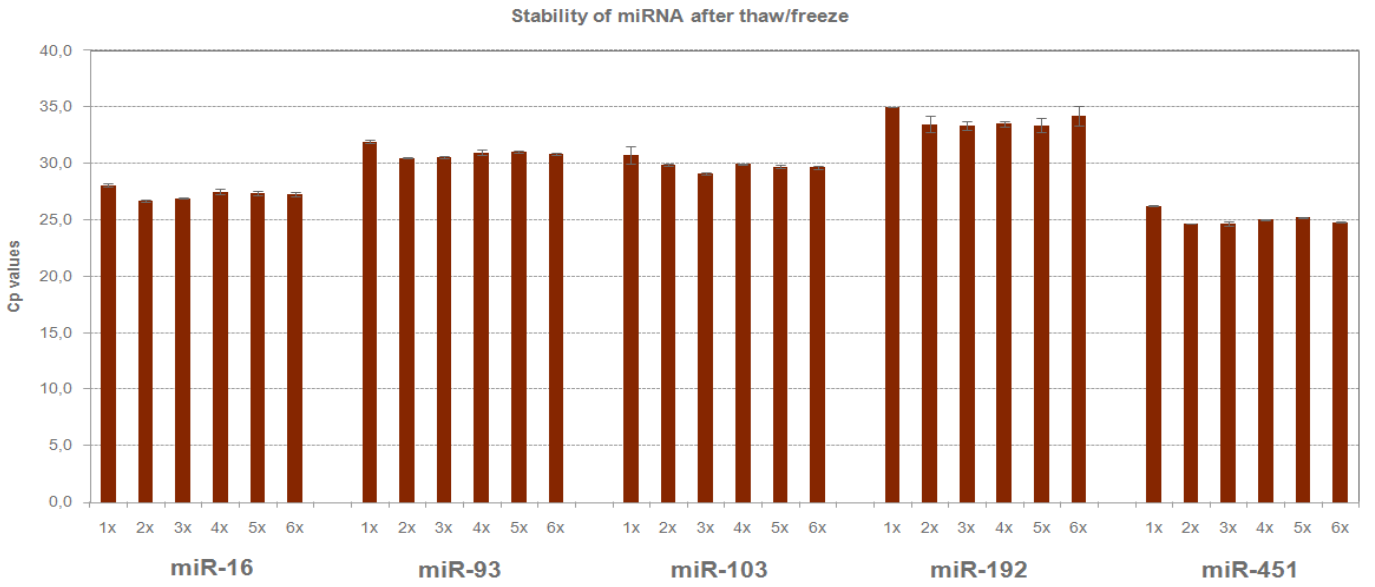


Place, date, unit, occasion etc.
Slide 1

Clinical QC of miRNA stability in blood derived Plasma

UNIVERSITY OF COPENHAGEN FACULTY OF LIFE SCIENCES

Stability of miRNAs in plasma subjected to multiple freeze thaw cycles prior to extraction.



Place, date, unit, occasion etc.
Slide 2

microRNAs as a source of biomarkers in blood

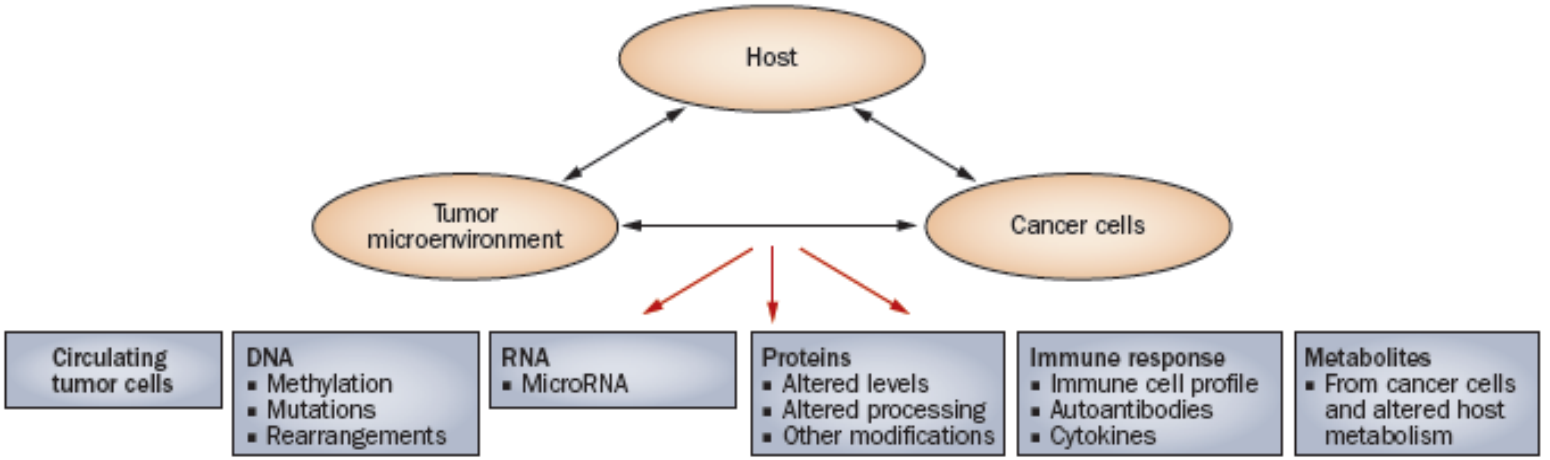
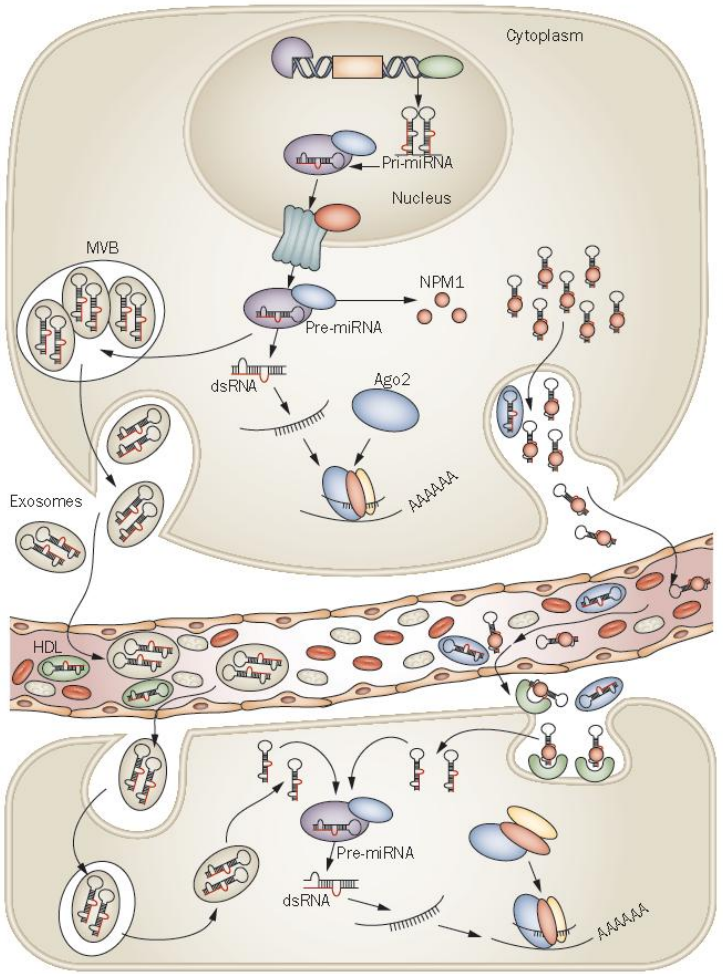


Figure 1 | Sources of blood-based biomarkers. Interactions between cancer cells, tumor microenvironment and the host result in the release of circulating cellular and molecular elements that can serve as rich sources of circulating biomarkers.

S Hanash et al Nat Rev Clin Oncol. 2011 Mar;8(3):142-50.

microRNAs as a source of biomarkers in blood



REVIEWS

MicroRNAs in body fluids—the mix of hormones and biomarkers

Maria Angelica Cortez, Carlos Bueso-Ramos, Jana Ferdin, Gabriel Lopez-Berestein, Anil K. Sood and George A. Calin

7248–7259 Nucleic Acids Research, 2010, Vol. 38, No. 20 Published online 7 July 2010
doi:10.1093/nar/gkq601

Export of microRNAs and microRNA-protective protein by mammalian cells

Kai Wang, Shile Zhang, Jessica Weber, David Baxter and David J. Galas*,†

Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma

Jason D. Arroyo[a], John R. Chevillet[a], Evan M. Kroh[a], Ingrid K. Ruf[a], Colin C. Pritchard[b], Donald F. Gibson[b], Patrick S. Mitchell[a,1], Christopher F. Bennett[a,c], Era L. Pogosova-Agadjanyan[d], Derek L. Stirewalt[d], Jonathan F. Tait[b], and Muneesh Tewari[a,d,e,2]

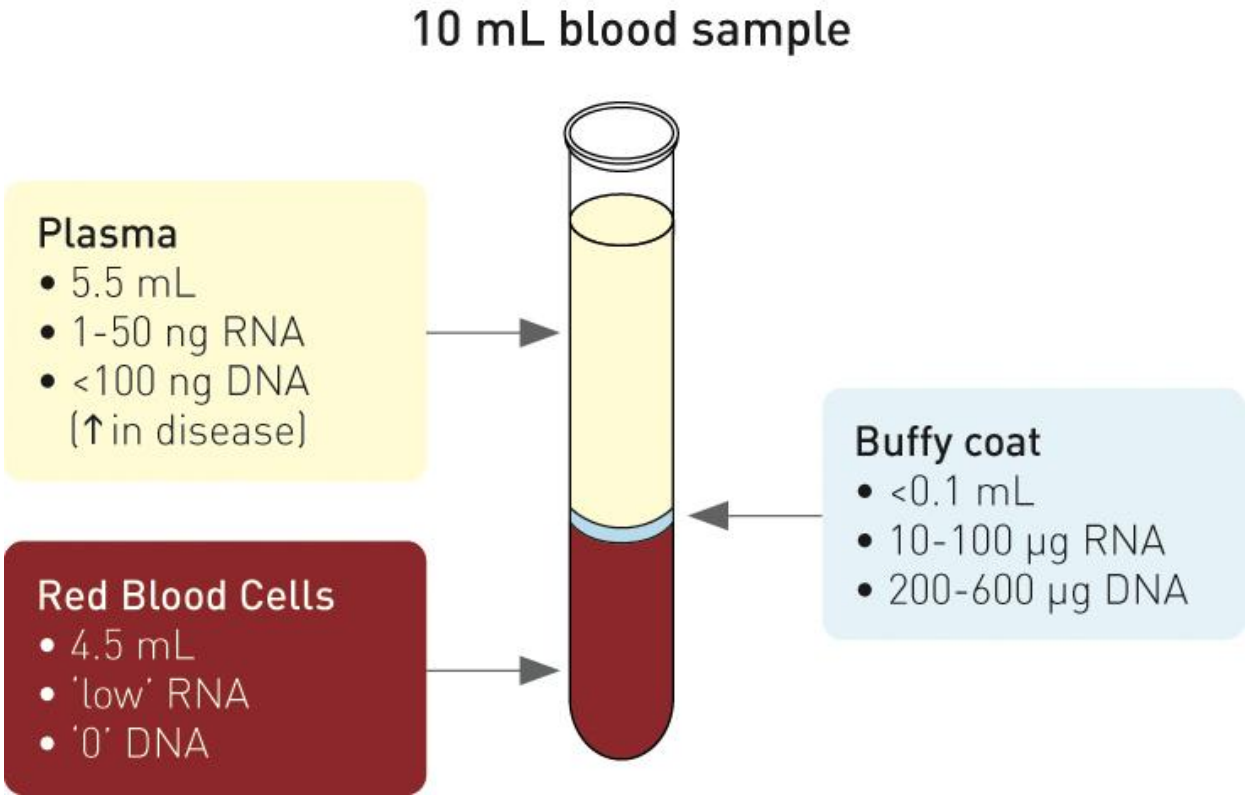
nature cell biology

MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins

Kasey C. Vickers[1,2], Brian T. Palmisano[1], Bassem M. Shoucri[1], Robert D. Shamburek[1] and Alan T. Remaley[1]

Cortez, M. A. et al. Nat. Rev. Clin. Oncol. 8, 467–477 (2011)

Limited clinical samples



miRCURY LNA™ Universal RT microRNA PCR - product overview

Ready-to-use panels (384-well), for full screening:

- Human, 742 microRNAs, 2 plates: 40 ng total RNA or 70 µl plasma/serum
- Mouse/Rat, 751 microRNAs, 2 plates: 40 ng total RNA or 70 µl plasma/serum

Custom Ready-to-use panels (96- and 384-well):

- Design your own Pick&Mix Panel – include only your microRNAs of interest

Individual primer sets, pre-designed and validated (human, mouse, rat)

- 1250 human, mouse and rat microRNA assays
- Reference gene primer sets (small RNAs)
- 1 pg total RNA is sufficient for reliable quantification

Custom LNA™ primers sets – design on-line (complex algorithm)

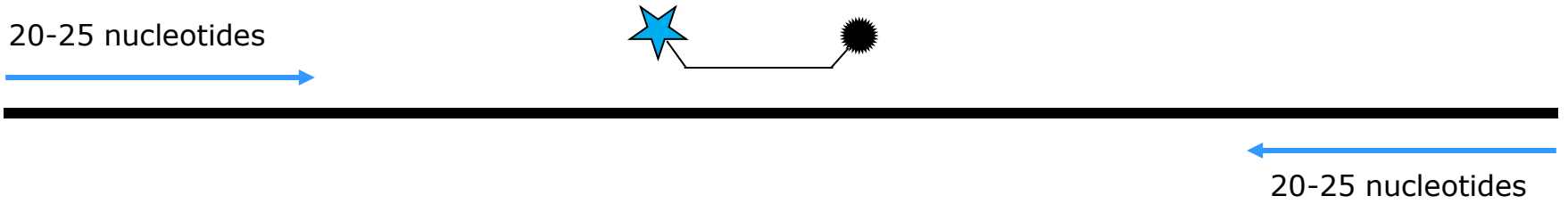
Reagents – optimized for use with microRNA primer sets:

- cDNA synthesis kit (86-32 rxns)
- SYBR® Green master mix (2.5 ml and 25 ml)

Exiqon's GenEx data analysis software

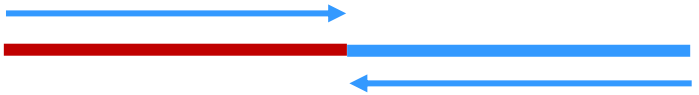
The challenge of miRNA qPCR:

Conventional qPCR



miRNA qPCR

miRNA qPCR



42

Strategies for microRNA qPCR:
PolyA tailing, Universal RT, SYBR® Green detection

PolyA tailing
and poly dT-tag RT

DNA forward primer and
universal reverse primer,
detection by SYBR® Green

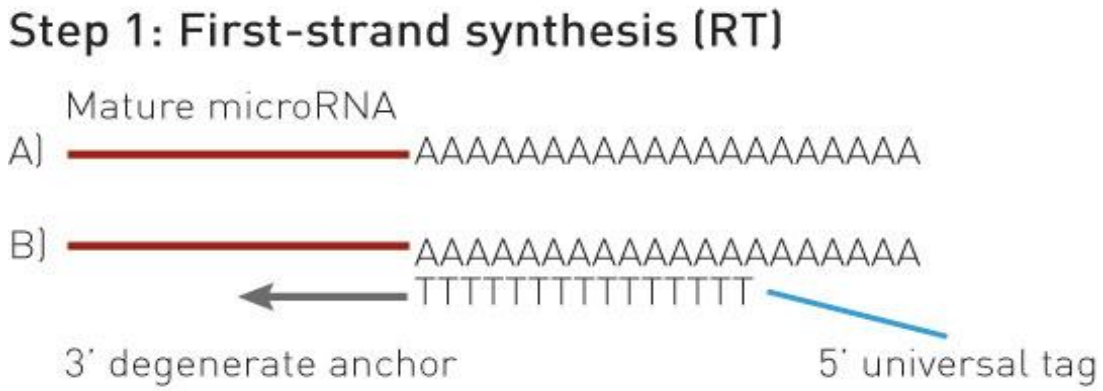
Advantages:

- One RT reaction
- Melt curve analysis is possible

Disadvantages:

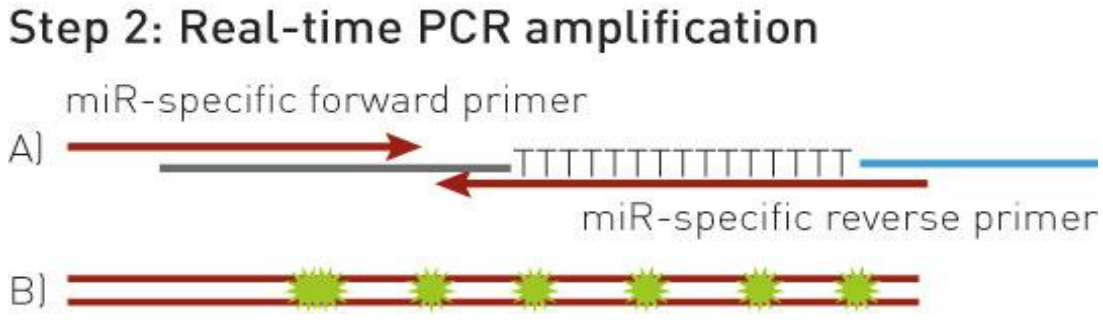
- Poor specificity: DNA primer close to length of microRNA = high background and poor sensitivity
- Many AT rich sequences will not be detectable
- Mismatches in the middle and 5’ end difficult to discriminate

Best of two worlds:
Universal RT with two microRNA specific PCR primers



Polyadenylation

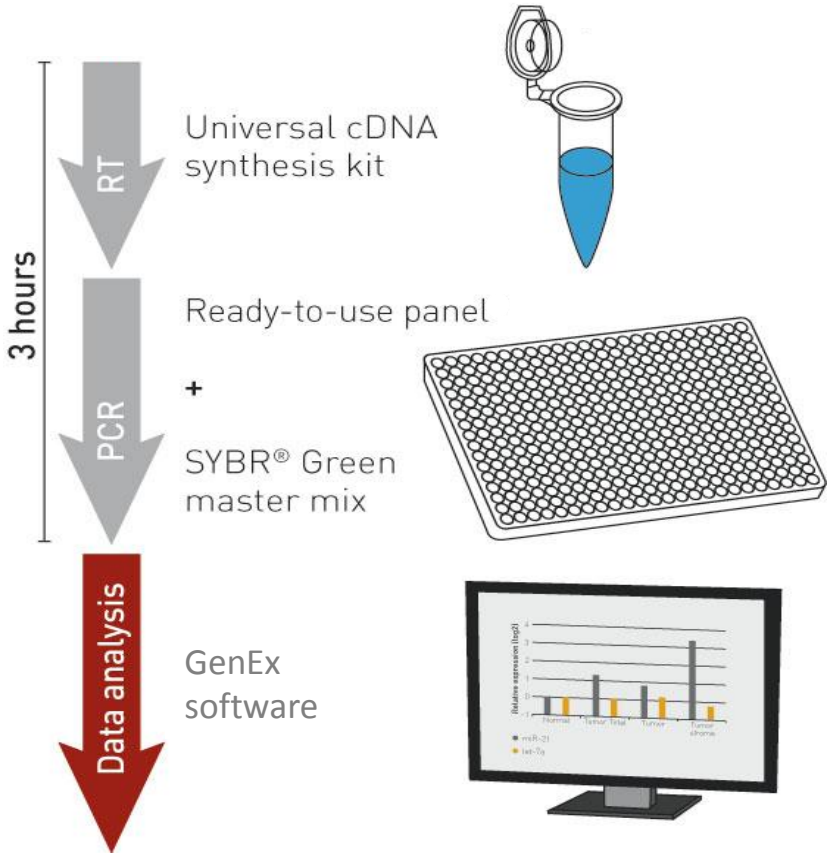
Reverse transcription



Two LNA™ enhanced microRNA specific primers

SYBR Green detection

Simple and rapid system for genome profiling of miRNA



1 RT reaction per sample

- Minimal sample input
- Few handling steps

1 one reaction per well

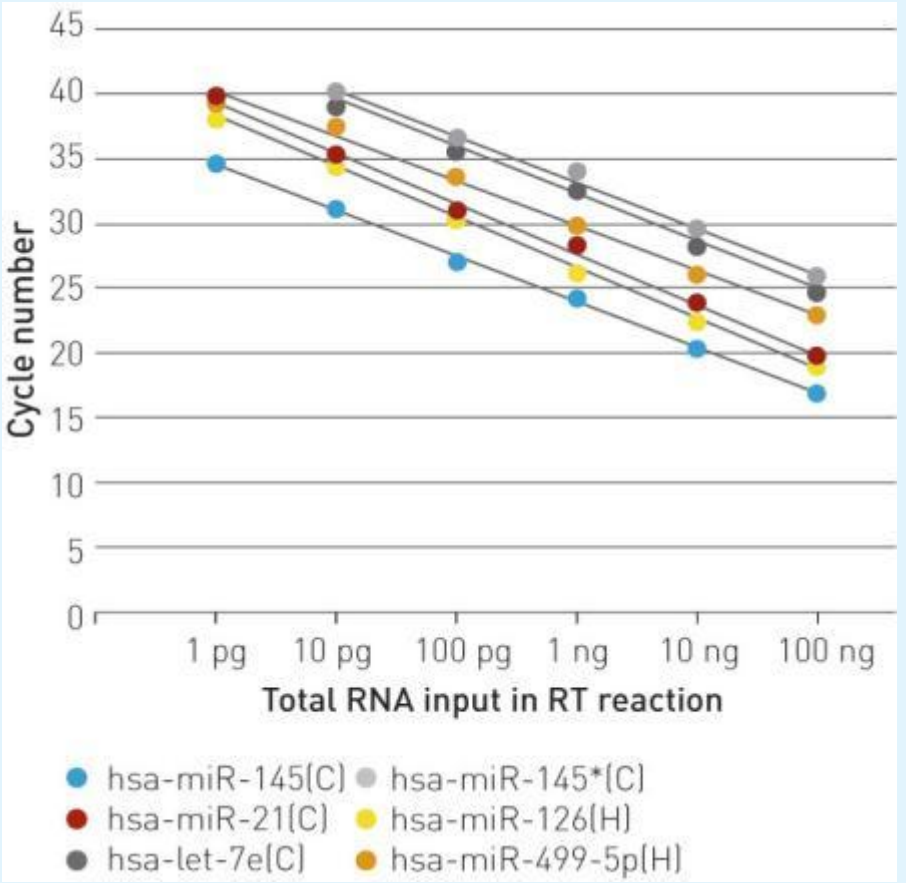
- Small reaction volume – 10 µl
- No assay pipetting – just add cDNA and SYBR® Green master mix

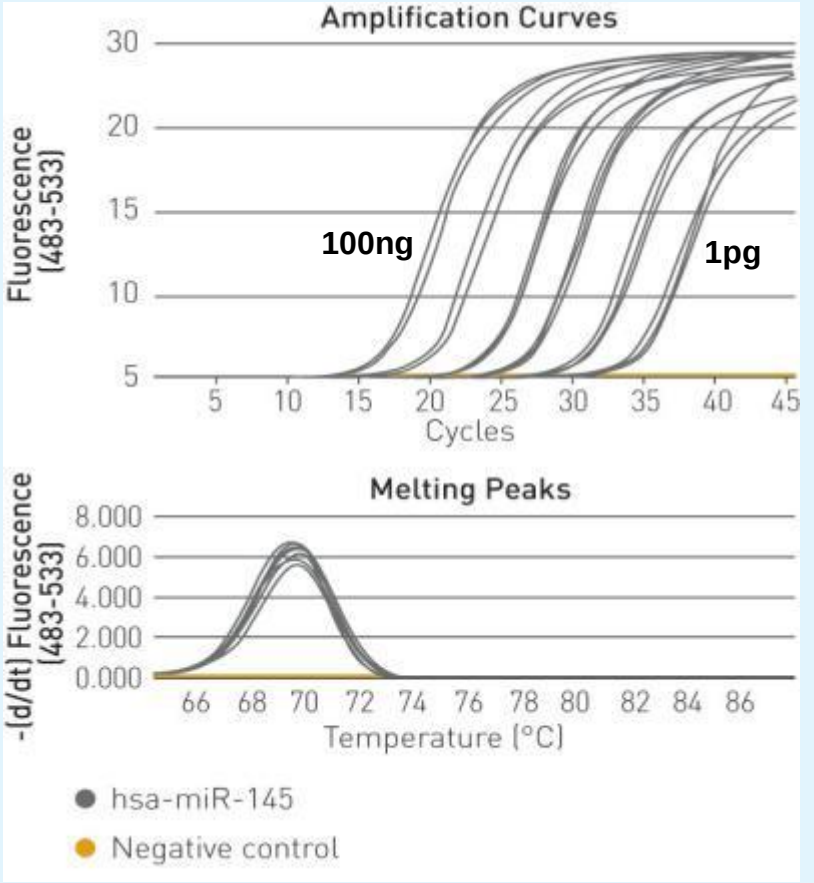
3 hours protocol

No need for pre-amplification

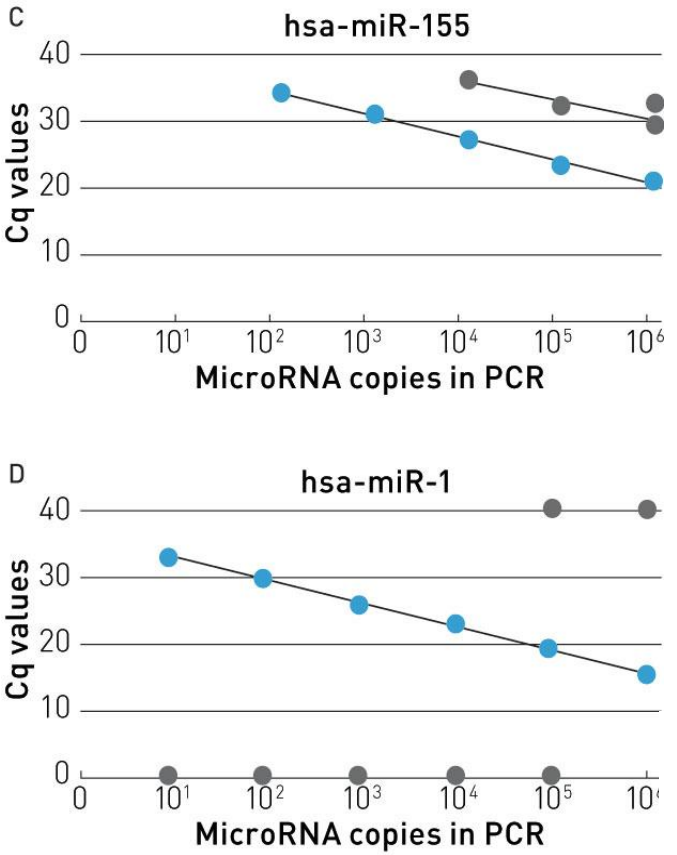
Plate layout file for direct import to GenEx

miRCURY LNA™ Universal RT microRNA PCR System





LNA™-enhanced primers enable detection of ALL microRNAs – even the AT-rich

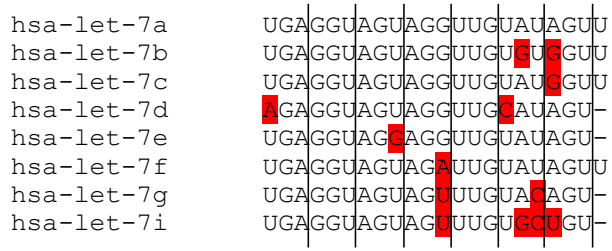


hsa-miR-155
61% AT content

hsa-miR-1
73% AT content

● LNA™ primers (Exiqon)
● DNA primers (competitor)

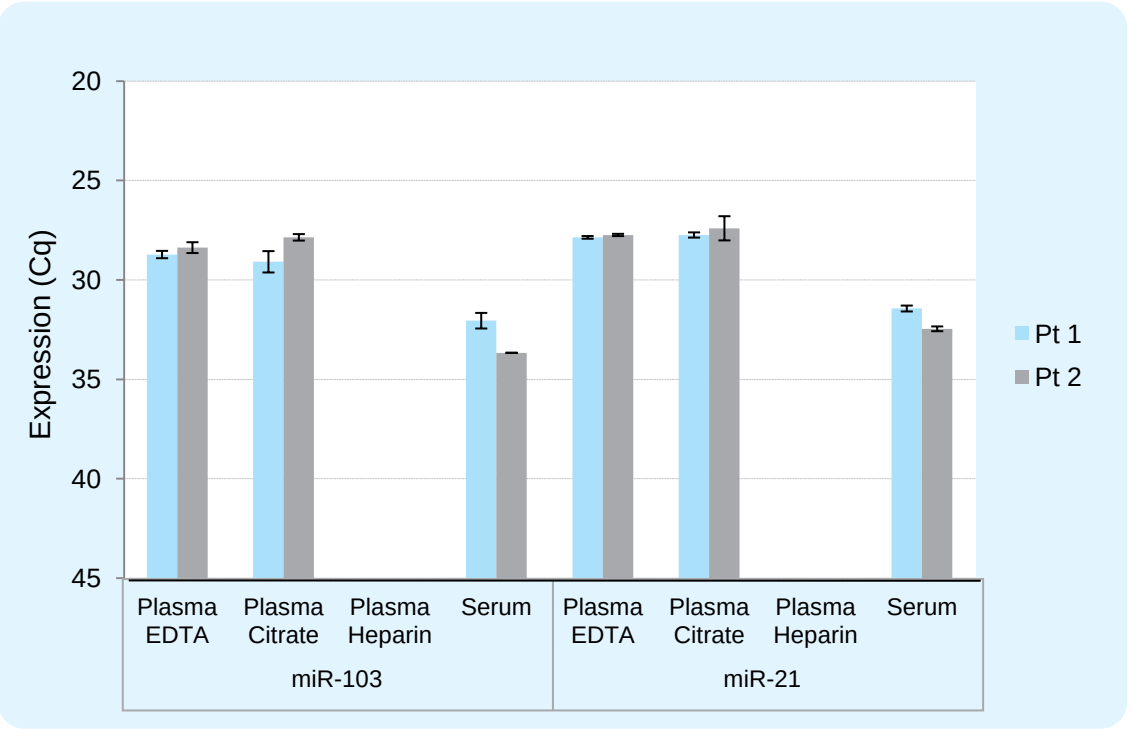
Excellent family member specificity



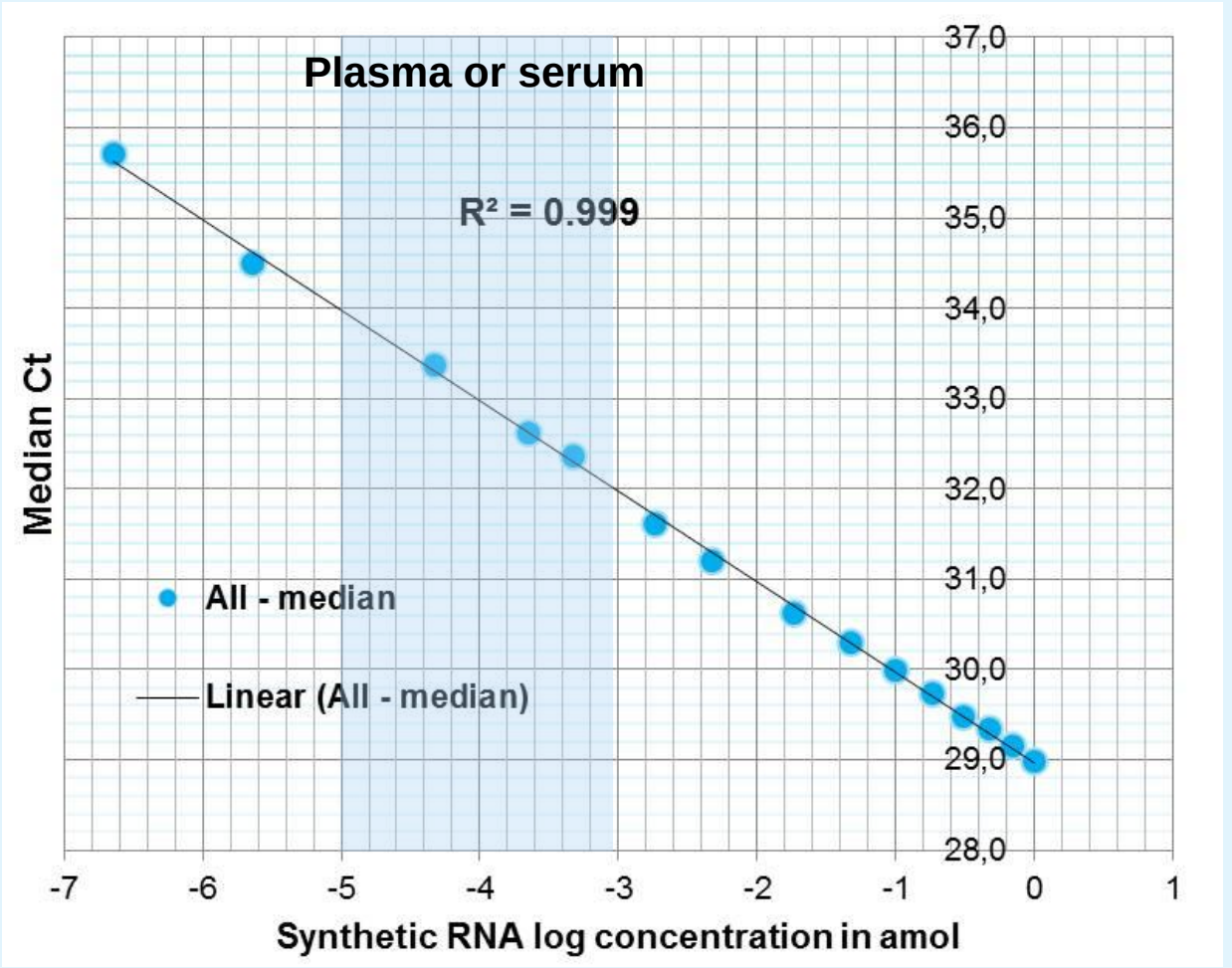
Let-7 family templates

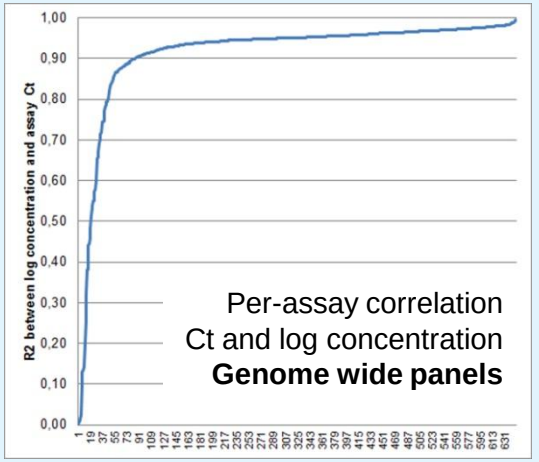
microRNA primer sets	hsa-let-7a	hsa-let-7b	hsa-let-7c	hsa-let-7d	hsa-let-7e	hsa-let-7f	hsa-let-7g	hsa-let-7i
hsa-let-7a	100%	0.0%	5.2%	0.0%	0.0%	0.1%	5.1%	0.0%
hsa-let-7b	0.0%	100%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%
hsa-let-7c	1.2%	0.0%	100%	0.0%	0.0%	0.0%	0.1%	0.0%
hsa-let-7d	0.0%	0.0%	0.0%	100%	0.0%	0.0%	0.0%	0.0%
hsa-let-7e	0.4%	0.0%	0.1%	0.3%	100%	0.0%	0.0%	0.0%
hsa-let-7f	0.0%	0.0%	0.0%	0.0%	0.0%	100%	0.0%	0.0%
hsa-let-7g	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100%	0.0%
hsa-let-7i	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100%

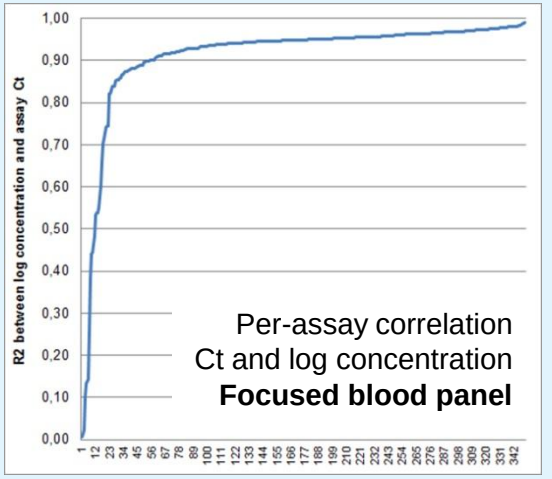
Blood collection tubes and subsequent effect on microRNA qPCR



Correlation between microRNA input and median Ct – near perfect linearity at very low concentrations as found in plasma and serum

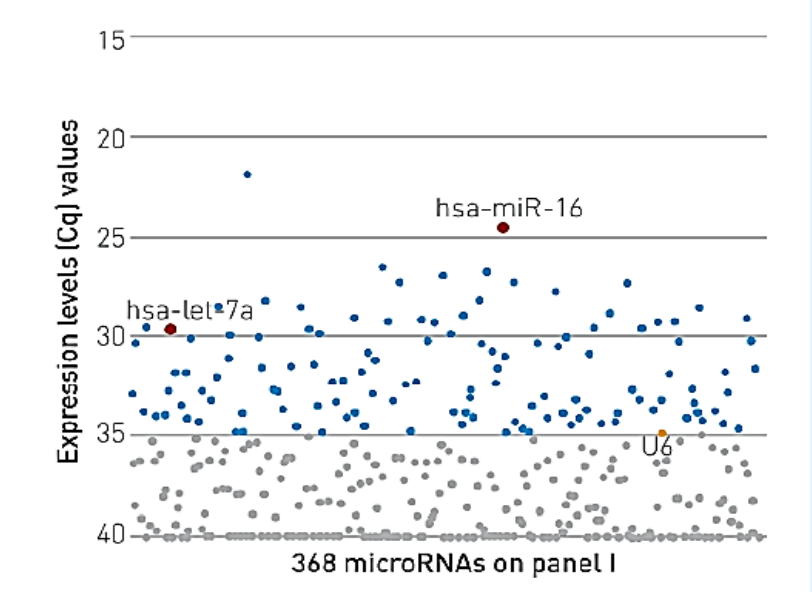


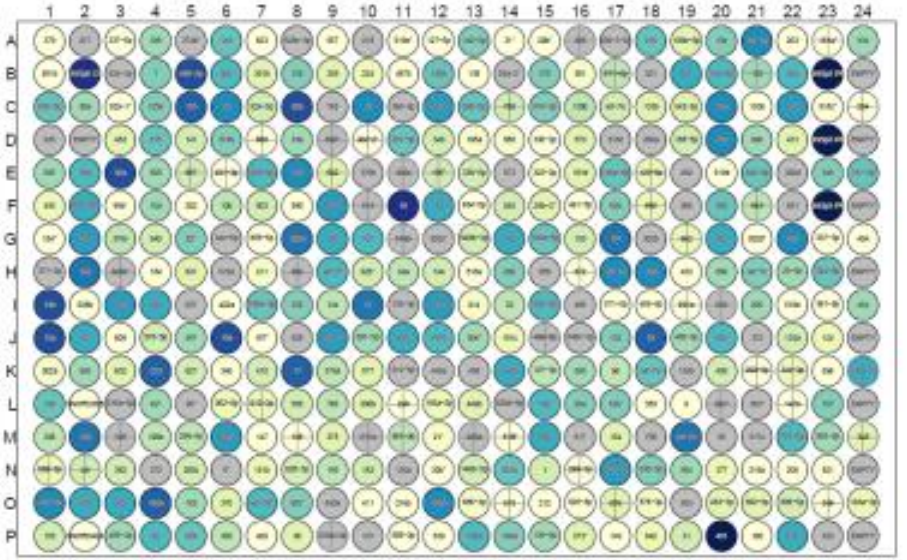




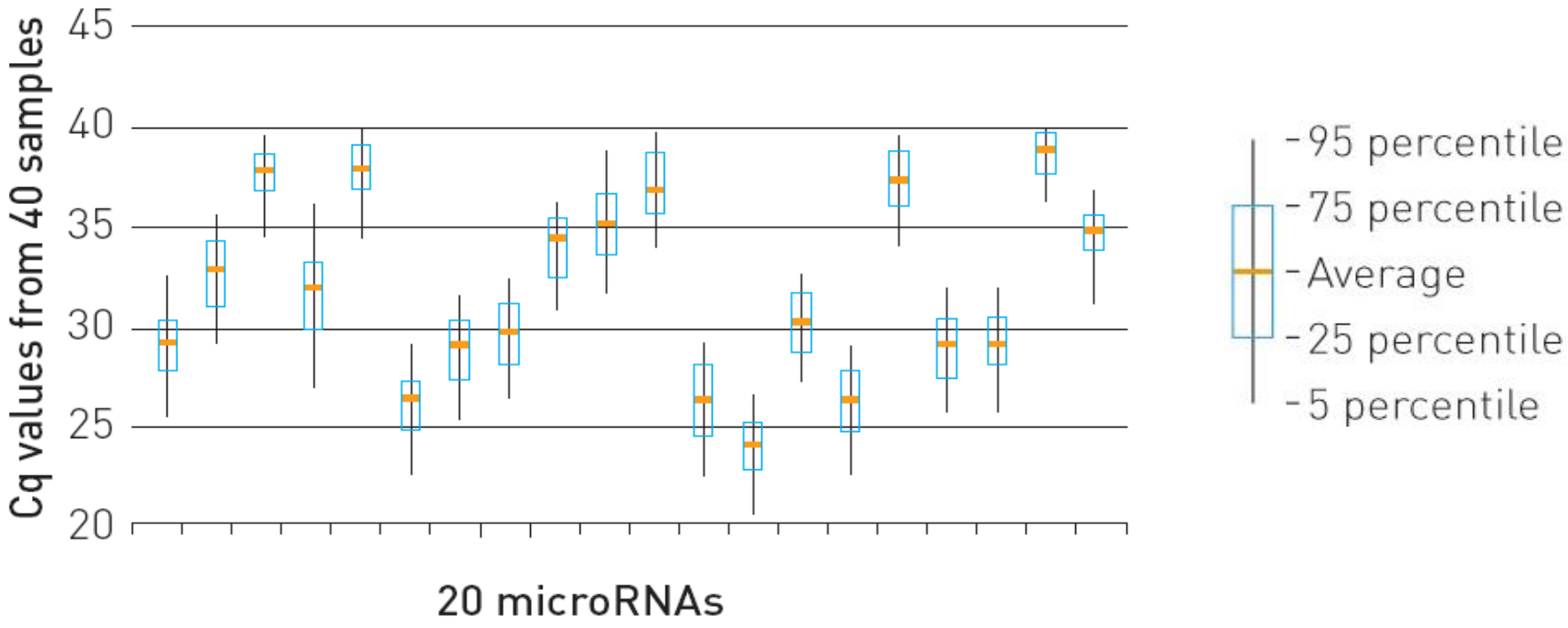
Genome-wide microRNA profiling based on serum

Profiling of microRNAs from 35µl of blood derived serum – with no pre-amplification

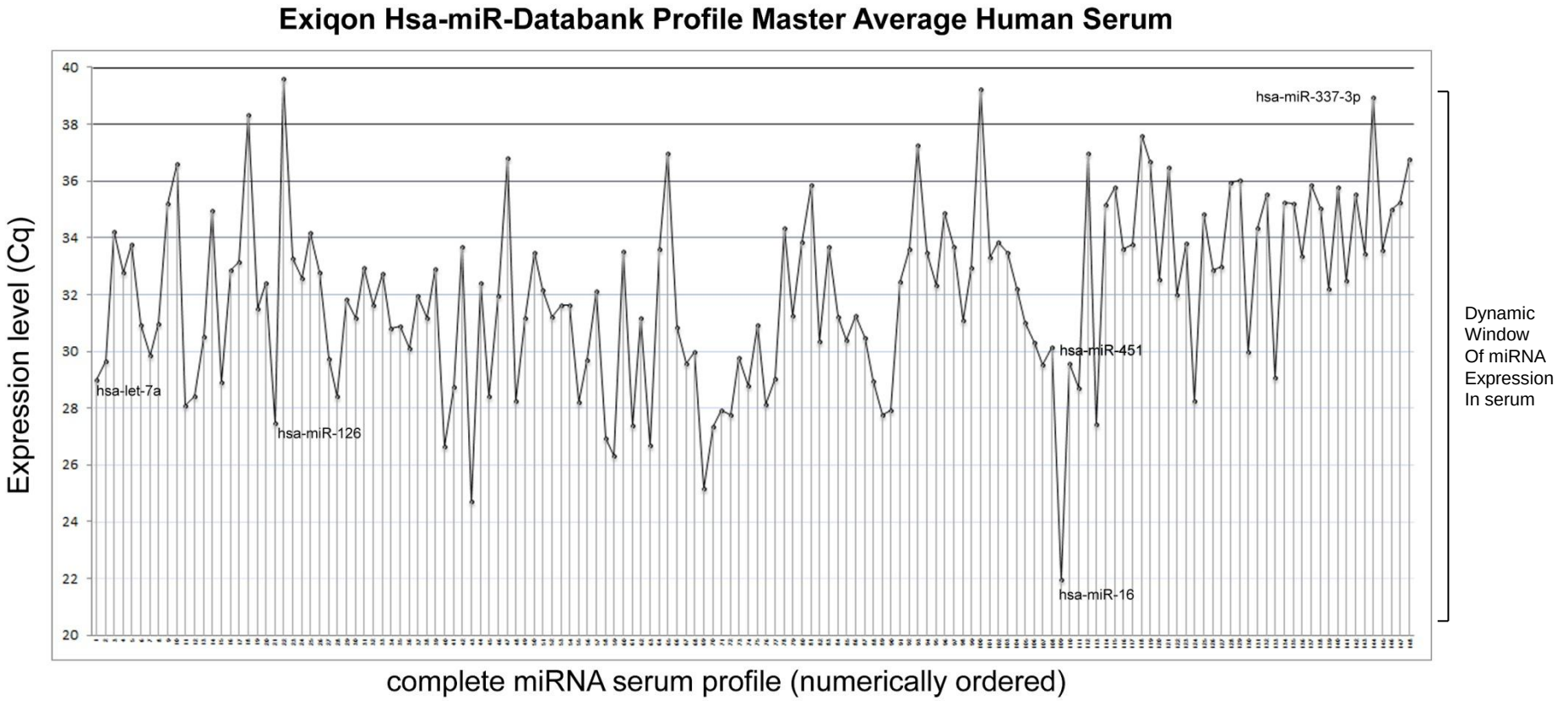




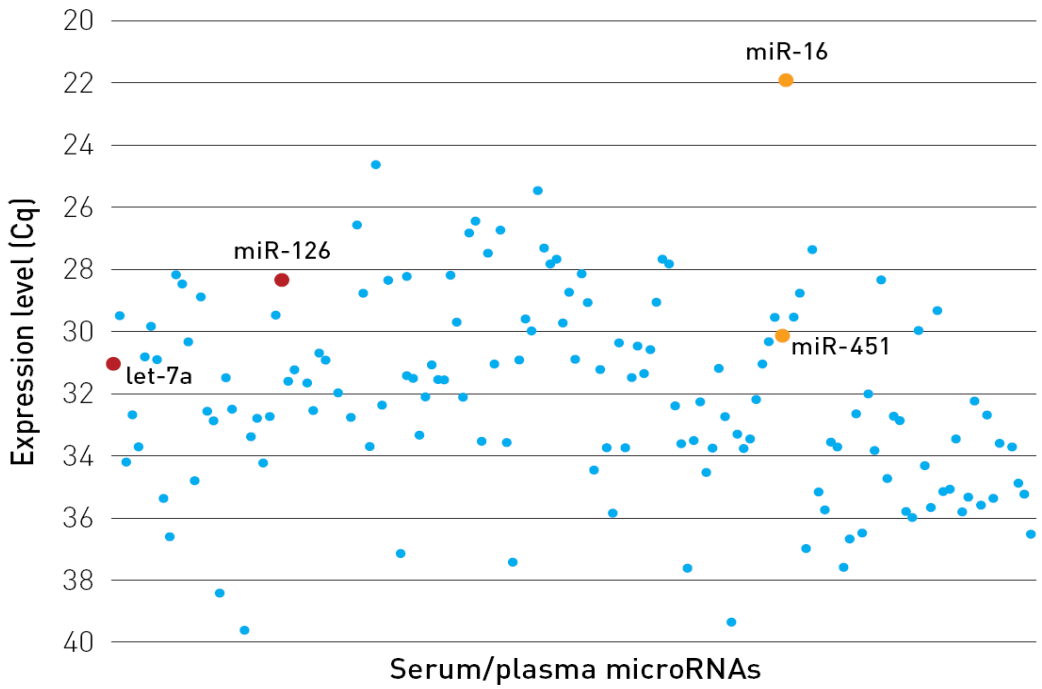
miRNA levels varies between healthy individuals, but within discrete ranges



QC Control: 490,368 PCR data point sample integrity check



Serum/plasma focus panel - 175 assays



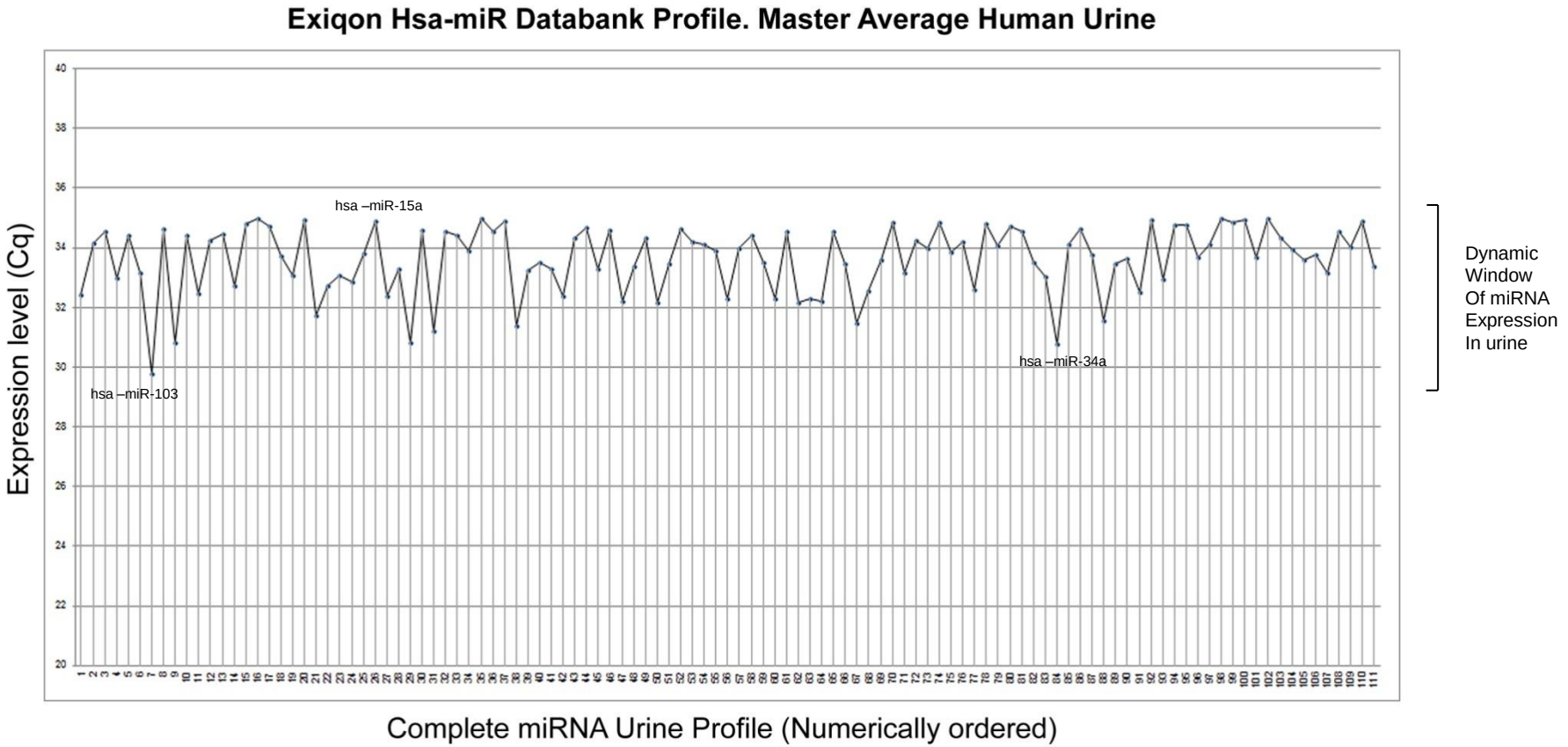
Profile all relevant serum/plasma microRNA from 20 µl serum/plasma

96-well
(1 panel in 2 plates)

384-well
(1 plate with 2 panels)

All the relevant controls

Human Urine microRNA Profile



Dynamic Window Of miRNA Expression In urine

- How to study microRNAs – introduction to LNA™
- miRCURY LNA™ microRNA arrays
- Early diagnosis of cutaneous T-cell lyphoma
- Detection of microRNAs in clinical samples and diagnostic development using LNA™ Universal RT microRNA PCR
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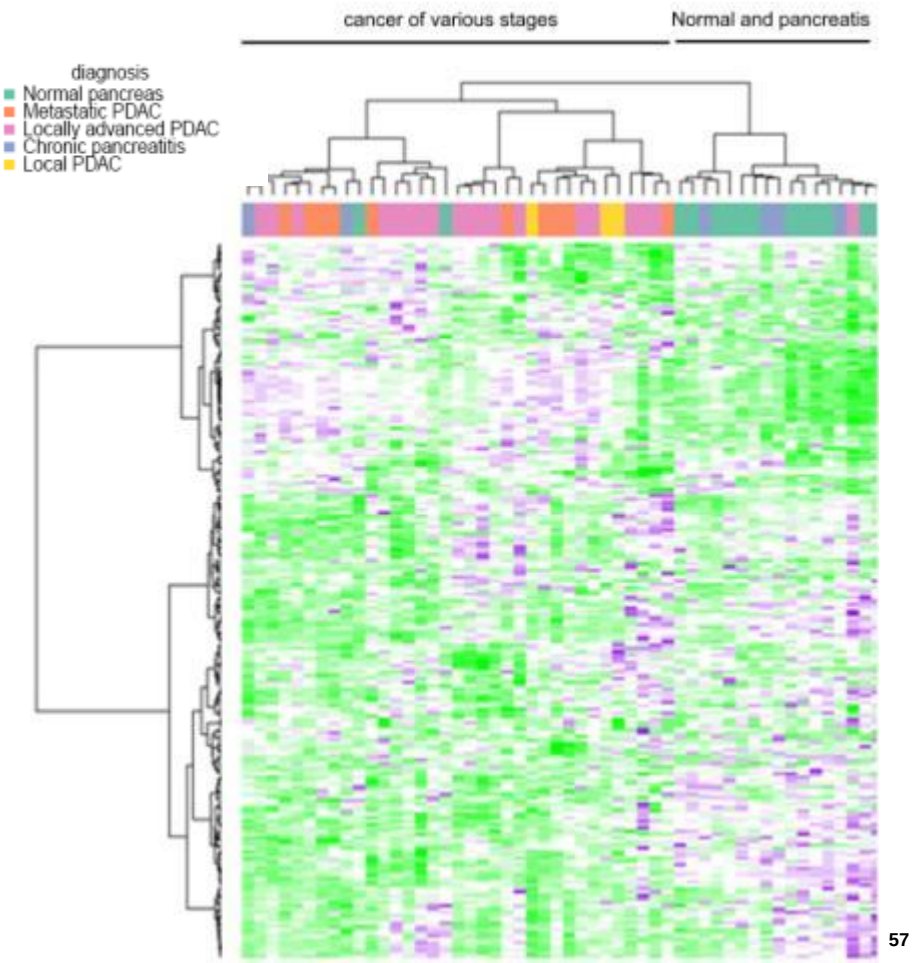
EXIQON
Seek Find Verify

Detection of early-to-late stage pancreas cancer in blood plasma

The experiment

- 30 pancreas cancer:
  - 3 local
  - 19 locally advanced
  - 8 metastatic
- 6 chronic pancreatitis
- 14 normal pancreas
- **Plasma** samples
- Whole genome microRNA profiling using LNA Universal RT microRNA PCR Panels
- Unsupervised clustering based on all detected miRNAs
- Normalized data

- Pancreatitis plasma clusters with normal plasma, and away from cancer plasma, irrespective of cancer stage

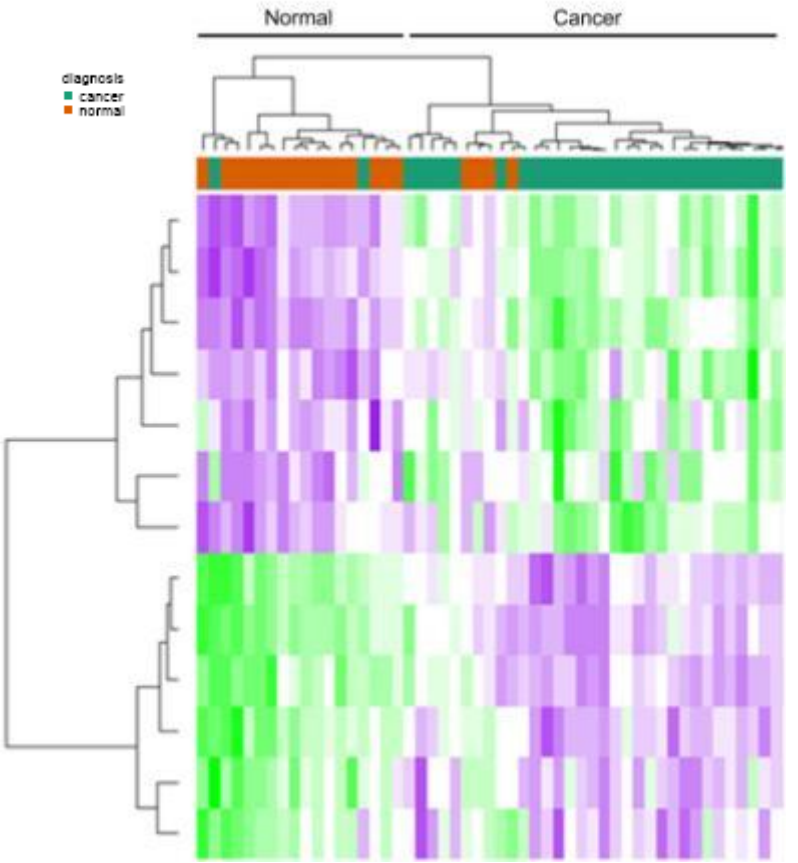


EXIQON
Seek Find Verify

Detection of early-to-late stage pancreas cancer in plasma

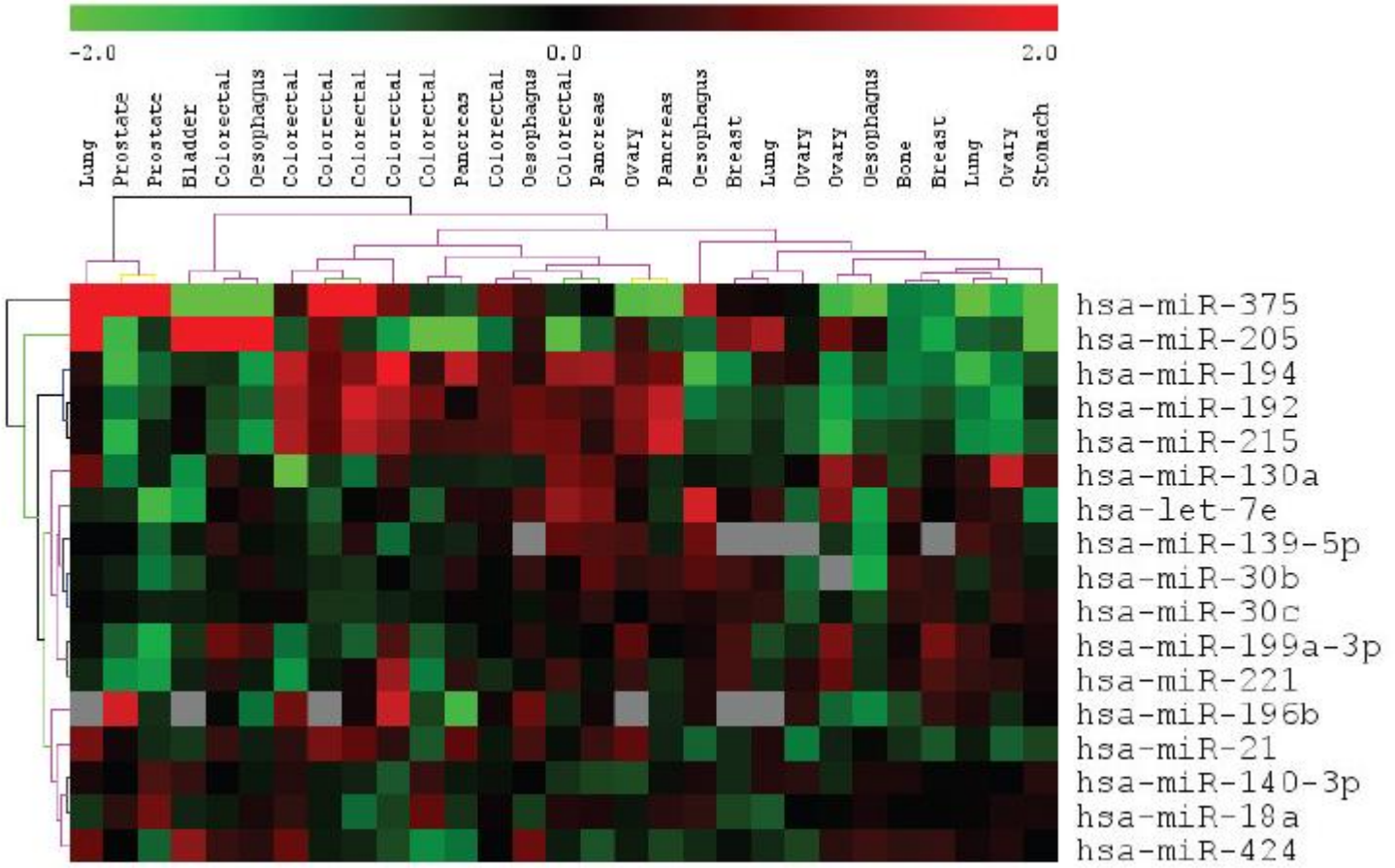
Cancer vs. normal in plasma

- Assumptions:
  - Pancreatitis and normal grouped as normal
  - All cancer stages grouped as cancer
- Clustering based on most significant miRNAs
- Promising separation between malignant and non-malignant pancreatic disease in plasma



EXIQON
Seek Find Verify

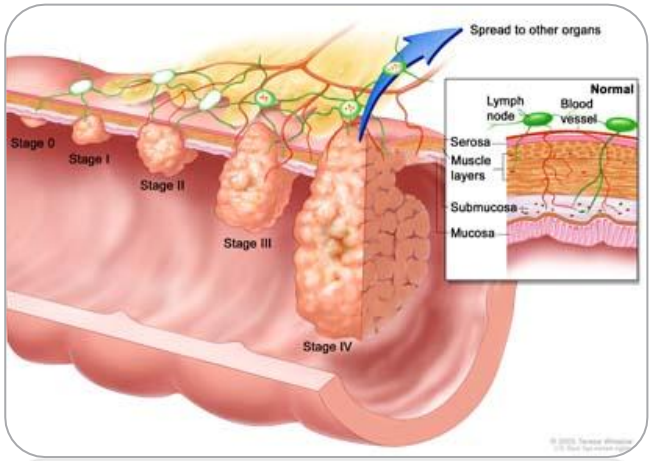
microRNA profiles can discriminate tumor type in serum samples



Unmet need for early detection test of Colorectal Cancer

Colorectal cancer

- Estimated new cases in 2010 in the USA:
  - Colon: 102,900
  - Rectum: 39,670
- Estimated deaths in 2010 in the USA:
  - Combined: 51,370
- **Early diagnosis results in resectable cancer with much improved prognosis**



Stage	5 yr relative survival	Treatment
0-I	93%	Surgery
II	80%	Surgery/discretionary adjuvant chemotherapy
III	58%	Surgery/adjuvant chemotherapy
IV	6.9%	Chemotherapy

Rationale for early detection test of CRC in blood plasma

Colorectal cancer (CRC) screening guidelines

For individuals between 50 and 75:

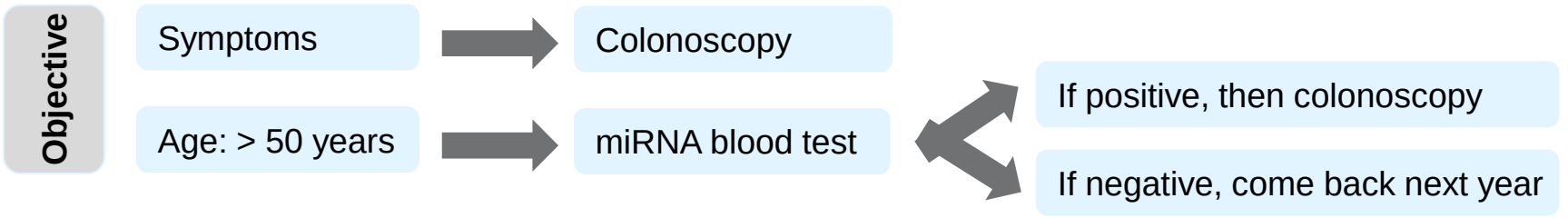
- Colonoscopy every 5 years (False-negative rate ~5%)

  or

- Annual FOBT (False-negative rate 20-75%)
- Poor compliance: <50% of population are screened

Large unmet need for minimally invasive screening assay for detection of CRC

Proposed application of early detection blood test in Colorectal cancer (CRC) screening



Work flow compatible with standard clinical procedures

Total lab time = 5 hours

	time = 1 hour	time = 1.5 hours	time = 2 hours	time = 0.5 hours
Plasma sample	RNA isolation	RT reaction	PCR reaction	Data QC and analysis.
• Standard Hospital procedures	• RNA isolation 200ul plasma required • Isolation of total RNA for from sample (no bias)	• Standard RT reaction. • Robust and reproducible	• PCR with LNA specific primers for detection of miRNAs	• Quality control • Data analysis and results • Diagnostic interpretation

- Assessible to standard hospital protocols
- No special handling/ storage requirements
- Low volume requirement

- FDA approved technology
- Conventional qPCR
- No black box algorithms
- Automatable

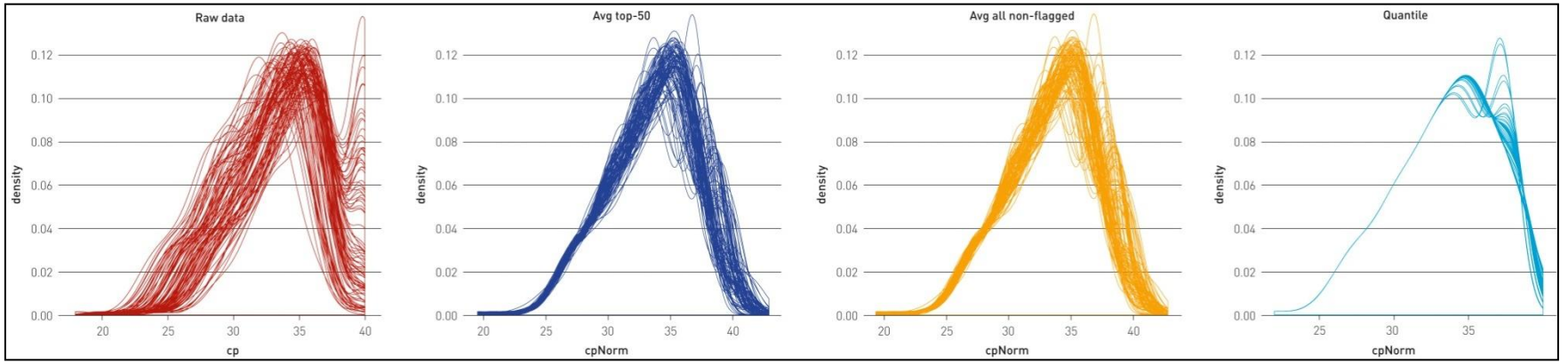
Normalization of qPCR data is not straightforward

Normalization of qPCR data is necessary to make expression values comparable across samples

Normalization adjusts for technical biases:

- variable amount of sample input RNA
- variable sample input RNA quality
- variable assay efficiencies due to e.g. sample specific assay inhibition

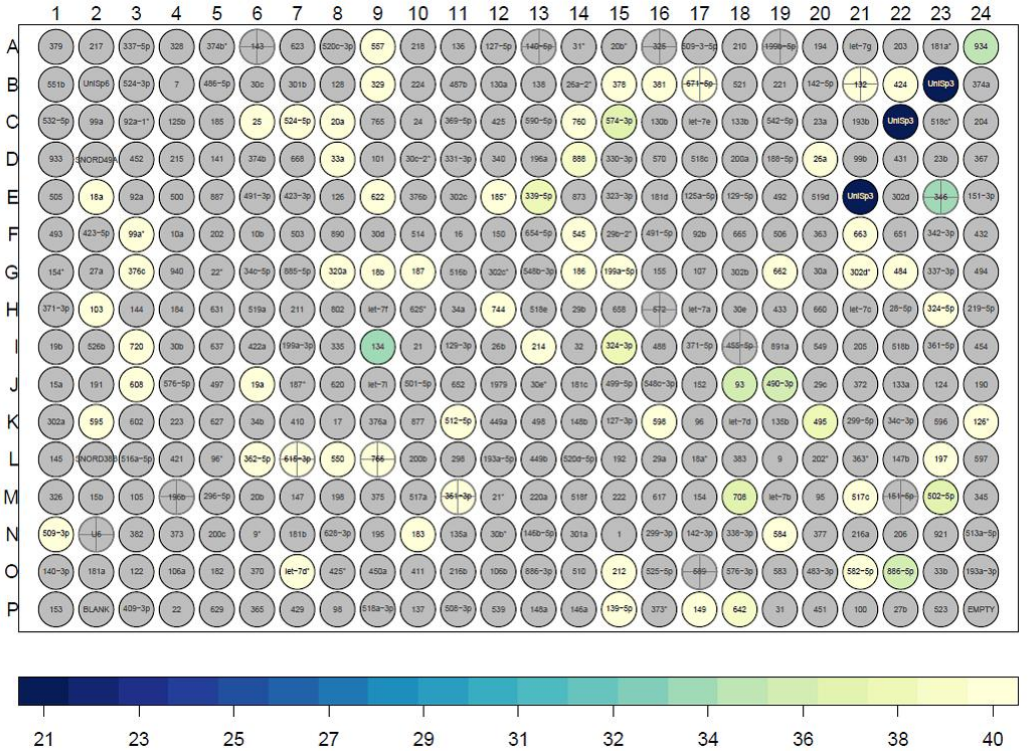
Prior assumptions about housekeeping genes across the samples of interest is not needed



Blank purification as a QC control of miRNA detection

Multiple controls on miRNA panel together with blank púrification produce good QC process

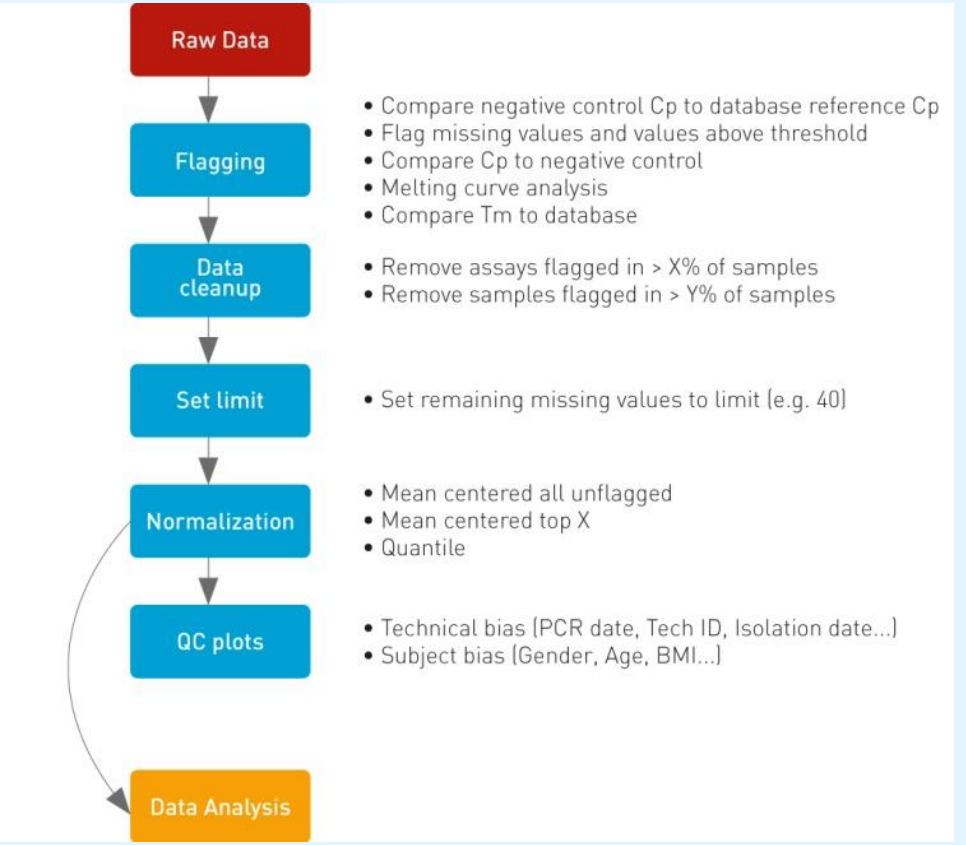
- Every system has a background and this needs to be assessed critically
- Water sample is exposed to the entire process to assess true background and thresholds.
- This is critical for the analysis of miRNAs in serum and other bio-fluids where levels are very low.



Extensive QC and data analysis pipeline

QC pipeline

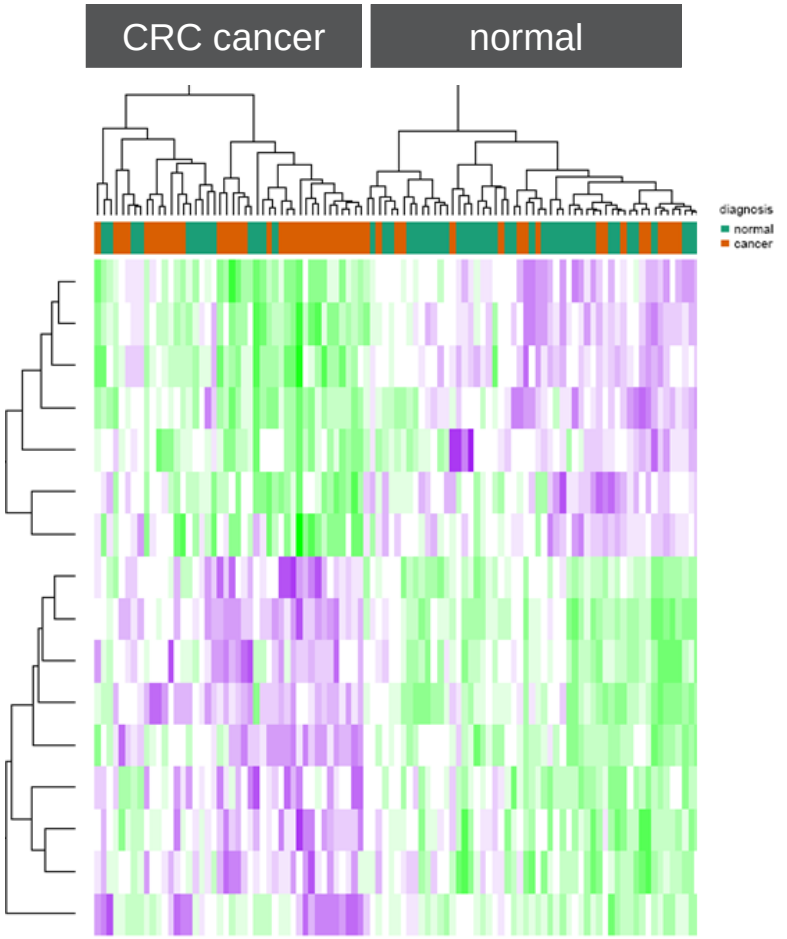
- Automated, user-guided QC pipeline
- Data flagging and cleanup customized for qPCR platform
- Diverse normalization protocols implemented
- Comprehensive QC report
- Visualization of potential technical and sample biases
- Identify samples that may be affected by contamination from blood cells



Pre-screen: Profile differs in plasma from CRC patients and healthy controls

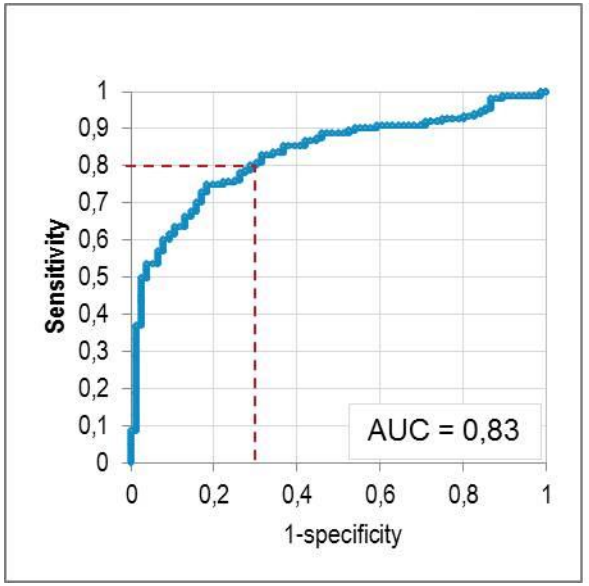
- 50 stage II colorectal cancer patients and 50 age- and sex-matched colonoscopy negative controls
- Plasma samples (pre-endoscopy)

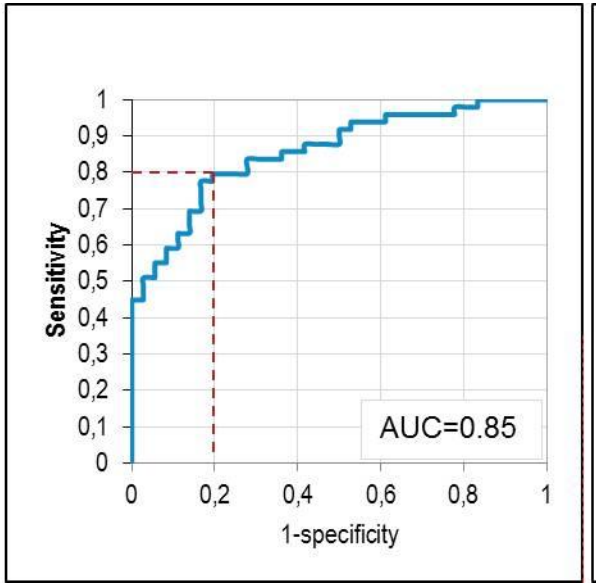
- Screening defined 378 candidate miRNAs present in plasma

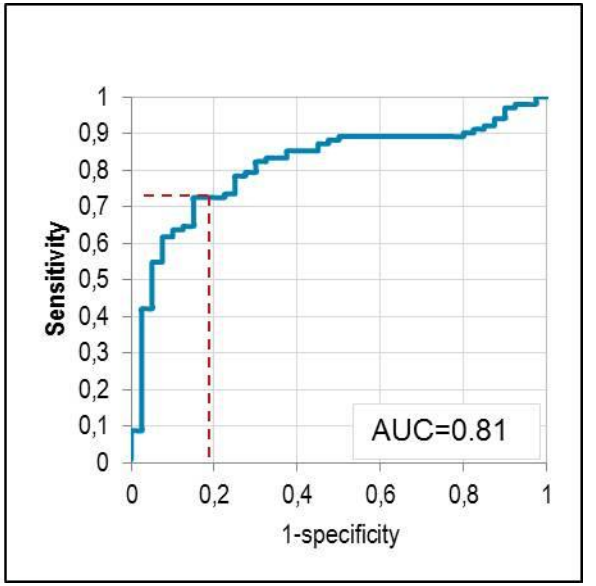


Focused panel of microRNAs in plasma may be used as biomarker for CRC

All Samples (n=227)	Hospital 1 (n=85)	Hospitals 2-5 (n=142)





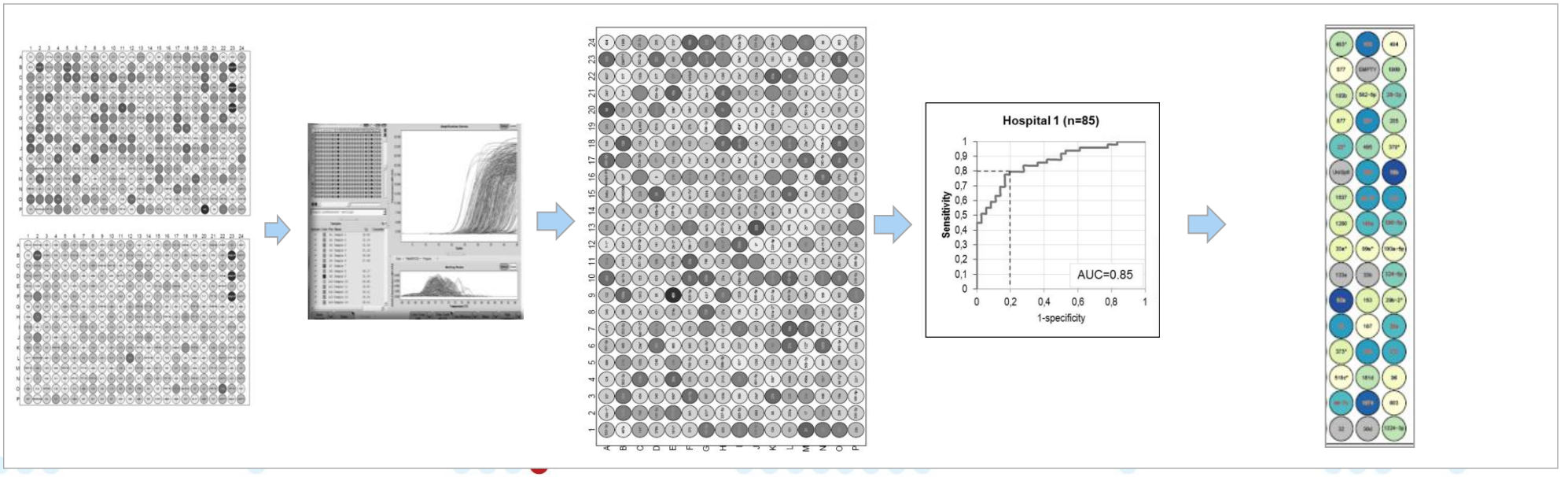


	All Samples	Hospital 1	Hospitals 2-5
Sensitivity *	75%	80%	73%
Specificity *	80%	78%	82%
(n) Cancer	151	49	102
(n) Control	76	36	40

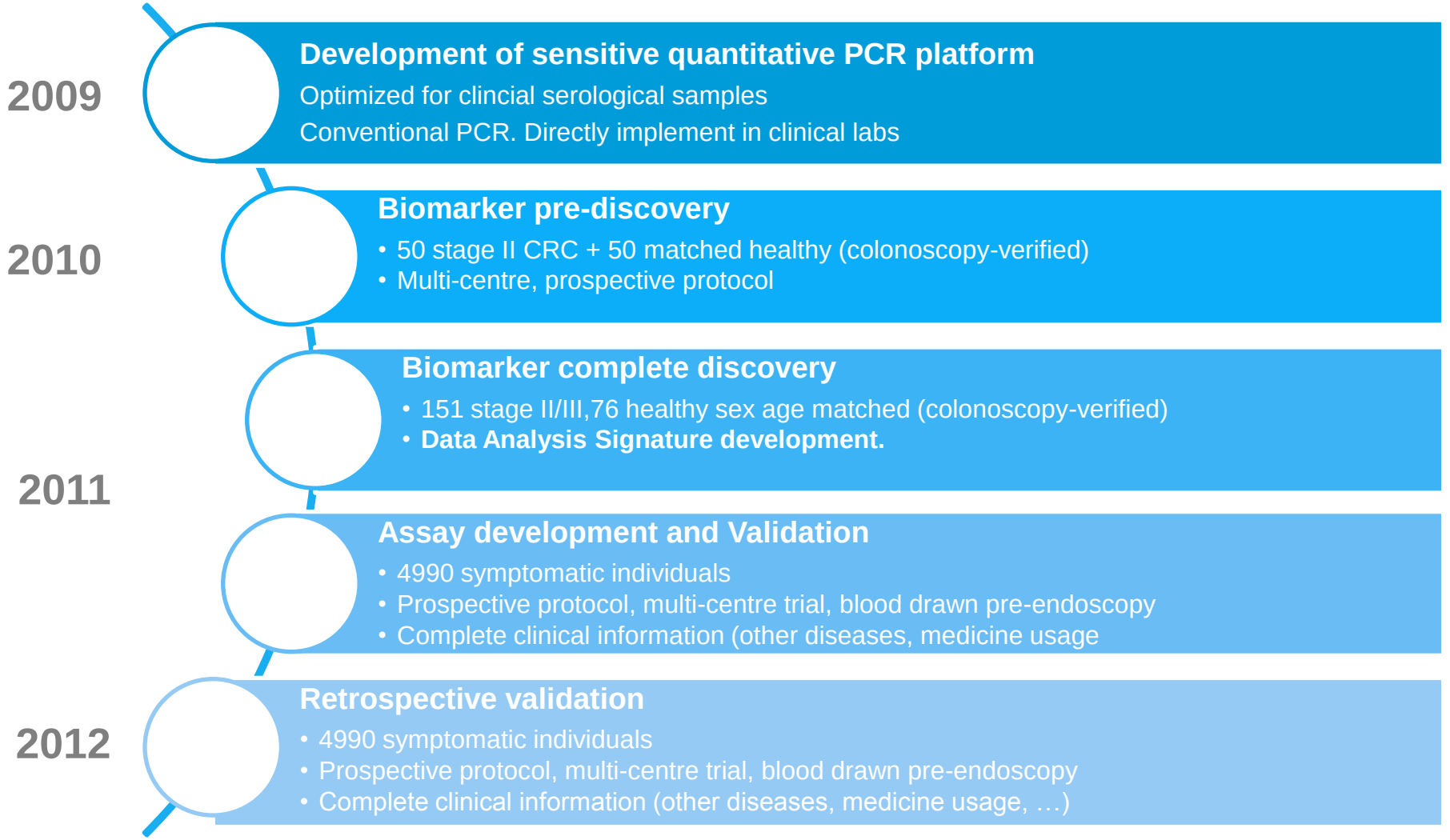
* The same cutoff score was applied on all samples in the study

Development of miRNA Early Detection Test of CRC in blood plasma

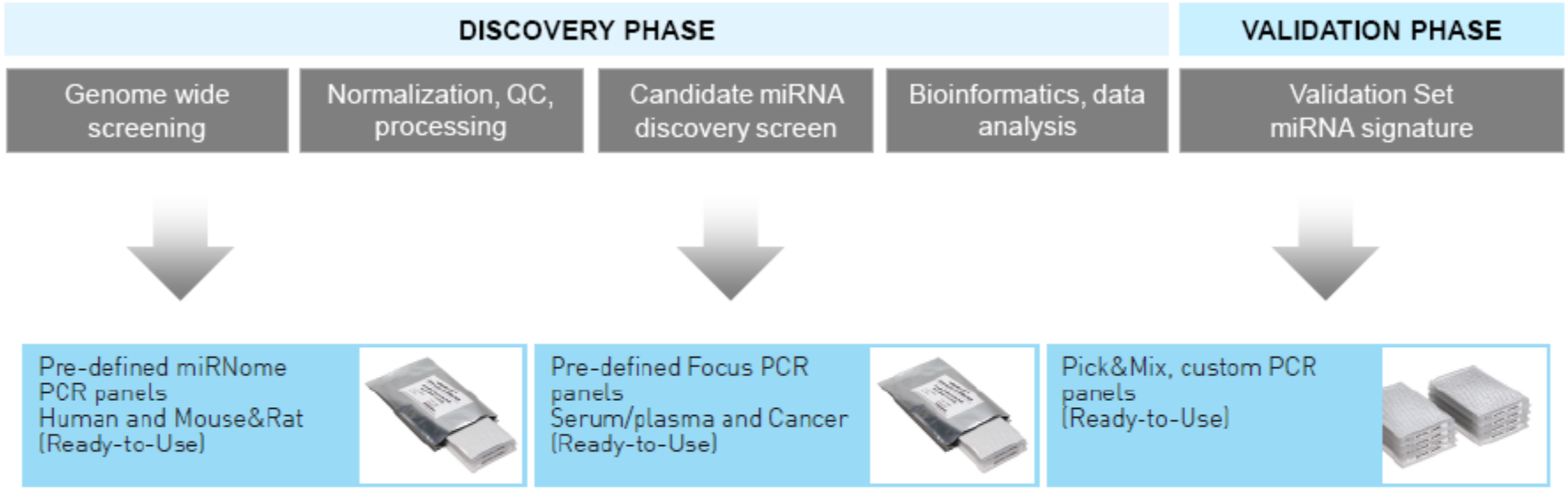
DISCOVERY PHASE				VALIDATION PHASE
Genome wide screening	Normalization, QC, processing	Candidate miRNA discovery screen.	Bioinformatics, data analysis,	Validation Set miRNA signature .
• 50 controls • 50 CRC patients • **742 miRNA screen** • **Genome wide**	• Multiple QC check • Data flagging • Normalization	• 76 controls • 151 CRC patients • **Focused Blood panel** • Multiple controls	• Data analysis • Quality control • ROC curve • miRNA selection	• 3000 patients (2011) • **Defined miRNA signature** • **Pick & Mix panel** • Multiple controls



miRNA Early Detection Biomarker from Discovery to Development



Biomarker discovery workflow and panel selection options



Serum/plasma Focus microRNA PCR Panel

- No need to spend time and money on miRNome-wide screening (Exiqon has done this for you)
- Comfortably start your focused screening with thoroughly selected serum/plasma microRNAs
- Move to Exiqon's Pick&Mix microRNA PCR Panel once microRNAs of interest are identified

Conclusions and future prospects

- Circulating microRNAs are promising biomarkers
- MicroRNA biomarker discovery should be included in early clinical studies

- microRNAs can be sensitively and reproducibly detected in serum / plasma without pre-amplification using the miRCURY LNA™ Universal RT microRNA PCR system
- Simple workflow allows test results from 100 µl of plasma within one day
- Flexible platform enables whole genome profiling and focused / customizable panels

- A plasma microRNA signature for colorectal cancer was developed and performs well in samples from independent hospitals
- Validation of the signature in a larger cohort is on-going

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Thank you for you attention!

miRNAs are promising novel biomarkers

Inserm/University Paul Sabtier

NOvember 22, 2011

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