

microRNA signatures for detection of early stage colon cancer in blood plasma

Carsten Alsbo, PhD

EXIQON
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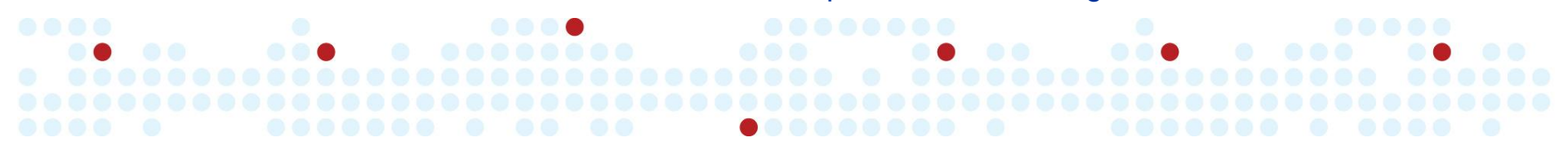
Presentation agenda

1. What are microRNAs?
2. Why study microRNAs?
3. How to study microRNAs – introduction to LNA
4. Case studies – microRNA biomarkers from human patient blood serum/plasma

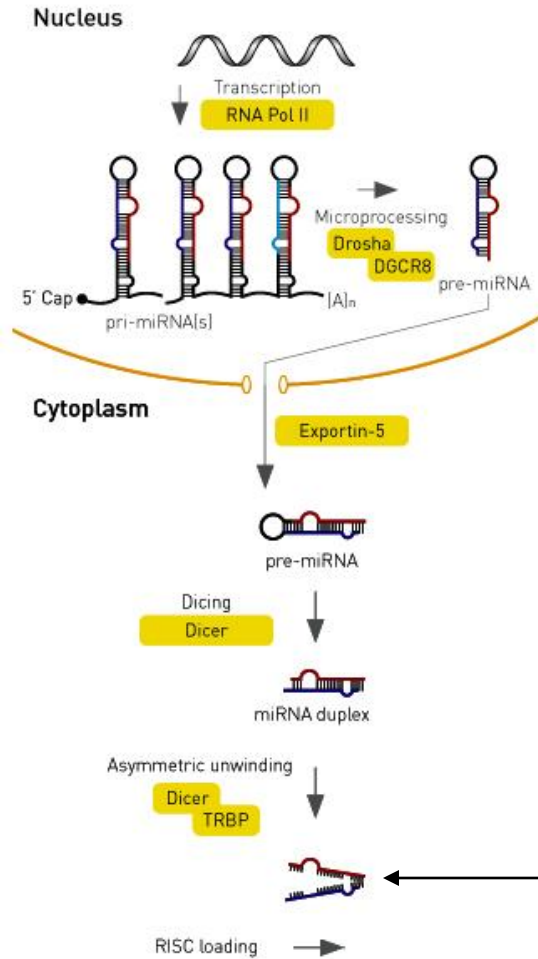
Six microRNA facts

1. Short non-coding RNA molecules of 19-22 nucleotides
2. Post-transcriptional regulators of mRNA
3. 1390 annotated human microRNAs* (1000 - 2000 predicted)
4. Regulate at least half 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)

MicroRNA is an exciting new dimension to gene regulation



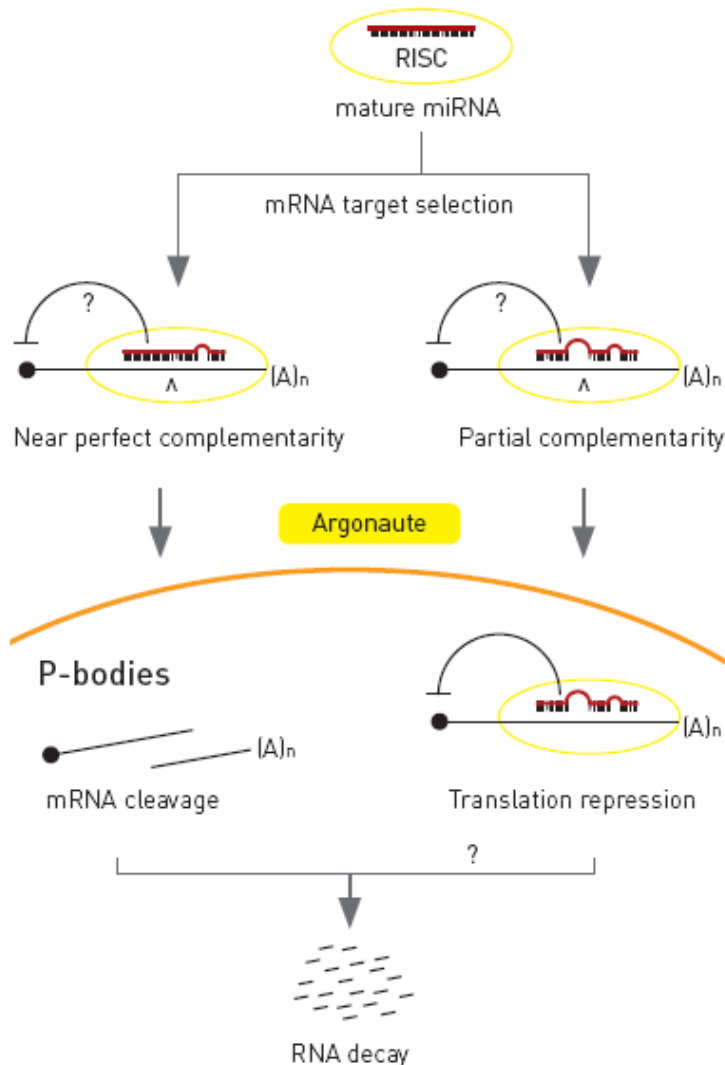
MicroRNA biogenesis



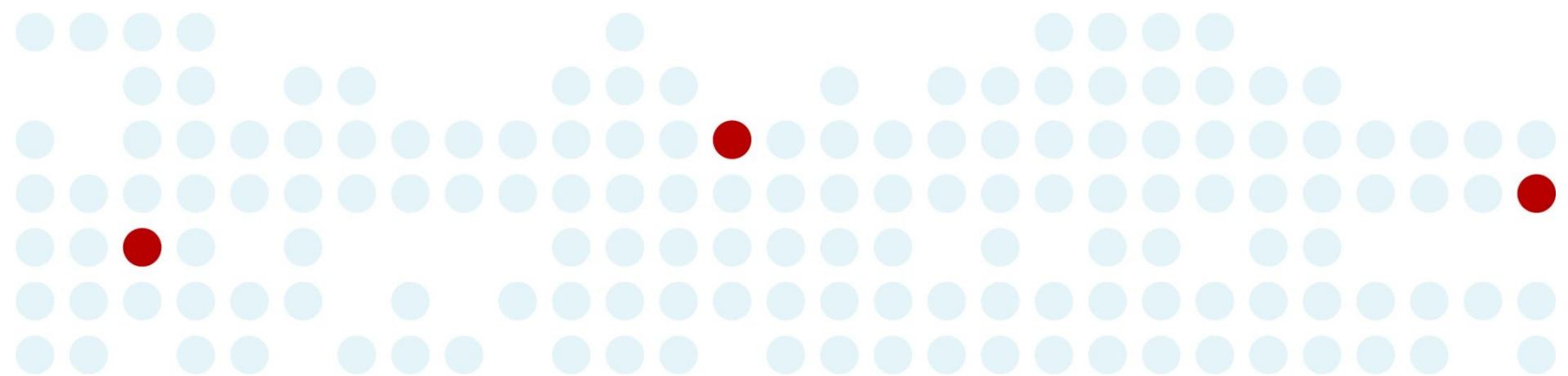
- Transcribed by polymerase II as long primary transcripts - pri-miRNA
- Cleaved by RNase III-type endonuclease Drosha to pre-miRNA (60-110 nt)
- Cleaved by Dicer into duplexes of 19-22 nucleotides
- RNA duplex is loaded into RISC and one of the strands is eliminated

One strand is the miR (or -5p), and the other is the miR* (or -3p)

microRNA mechanism(s) of action: Translational repression AND mRNA degradation



- Binding to 3' UTR of mRNA
- Repression of cap dependent (but not IRES dependent) translation
- Aggregation of mRNAs with mRNPs
- Localization of repressed mRNAs to P-bodies
- Outcomes:
 - Imperfect complementarity => translational repression / mRNA decay
 - Perfect complementarity => mRNA cleavage



Why study microRNAs?

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miRNAs PLAY A KEY REGULATORY ROLE

· **It has been documented that miRNAs are involved in:**

· **Cancer development**

- (“OncomiRs” or tumour suppressors)
- Brain, breast, colon, leukaemia, liver, lung, thyroid, pancreas.

· **Disease associations:**

- Type 2 diabetes
- Heart disease pathogenesis
- Psoriasis
- Alzheimer disease
- Atherosclerosis, postangioplasty restenosis, transplantation arteriopathy, and stroke
- Chronic pancreatitis
- Cardiomyocyte hypertrophy
- Host/virus interaction

· **Certain genetic disorders are likely to cause changes in miRNA regulation:**

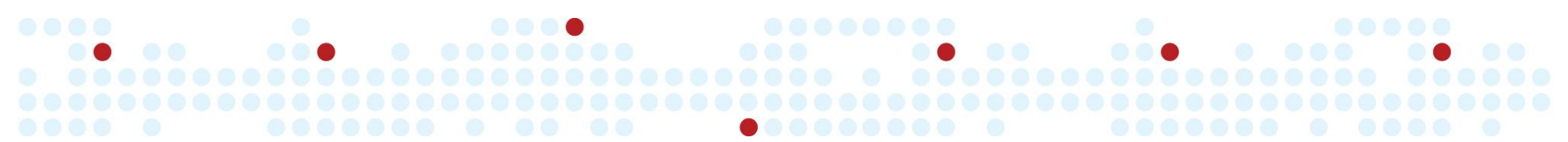
- Fragile X syndrome
- Spinal muscular atrophy pathogenesis

Development and differentiation:

- Adipocyte differentiation
- Cholesterol and fatty acid metabolism
- Blood
- Brain
- CNS
- Granulocytes
- Muscle
- Apoptosis
- Viral life cycle
- Toxicity
- Stem cell differentiation
-etc.

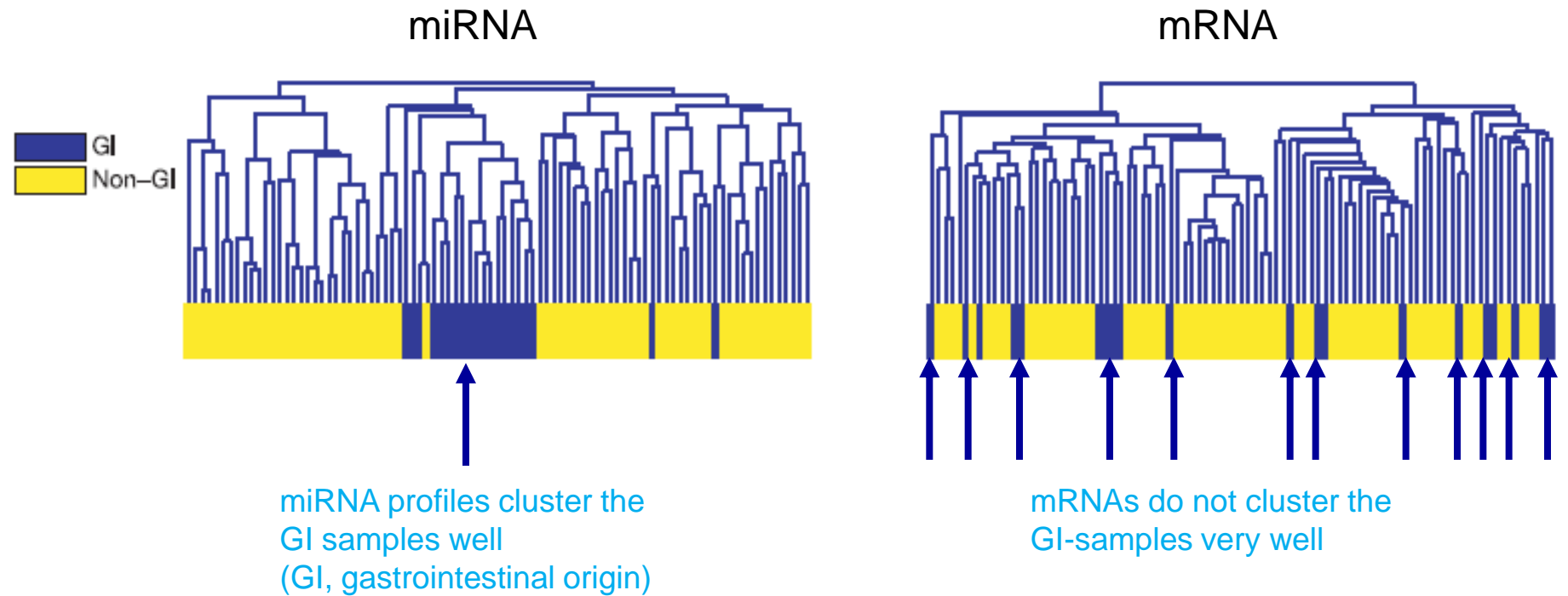
Classification Power:

- ✓ **Diagnostic**
- ✓ **Prognostic**
- ✓ **Predictive**



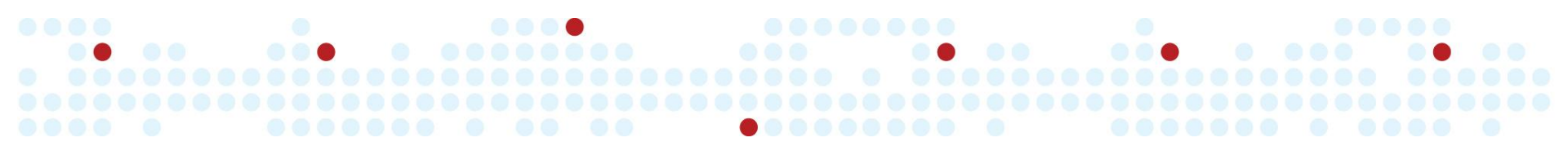
Classification – miRNA vs. mRNA

· microRNA profiles appear superior to mRNA for cancer classification



Reasons to use microRNAs as biomarkers

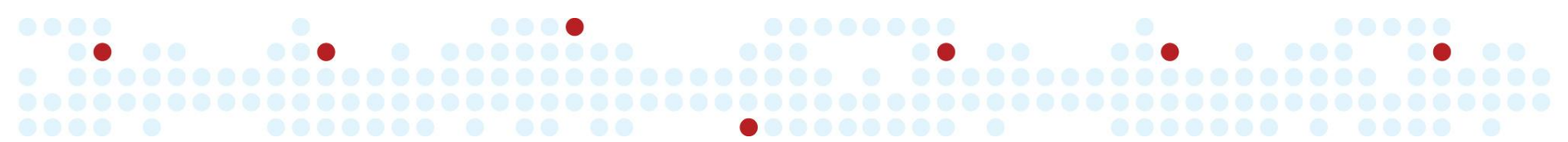
- Involved in pathway regulation & tissue differentiation
- Evolutionary conserved indicates important biological function
- Relatively small number of genes to profile
- Huge dynamic range (0 to 40,000 molecules per cell)
- Superior to mRNA for classification of cancers and other diseases
- Highly stable in sample preparation
 - MicroRNAs are much more stable in archival FFPE material than messenger RNAs – more likely to reflect the true nature of the sample or effect of the drug (less influenced by differences in sample collection)



Analyzing microRNAs – challenges using traditional DNA technology

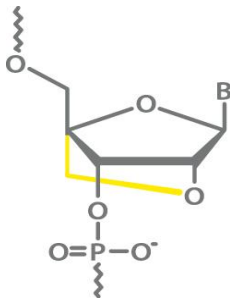
Feature	Challenge
Short and highly homologous sequences (single base differences)	Achiving high sensitivity Achiving sufficient specificity
Large variation in base composition in microRNAs (G/C rich & A/T rich)	Designing sensitive and specific probes within a limited sequence
Secondary structure	Limited accesibility of some microRNAs

Need for improved affinity and specificity



LNA™ technology overview and LNA™ Universal RT microRNA PCR System

LNA™



Technology allows for improved binding

- LNA is a bicyclic high affinity RNA mimic with the sugar ring locked in the 3'-endo conformation
- Increased T_m (T_m increases by 2 - 8°C per base)
- Improved mismatch discrimination
- High sensitivity and specificity in hybridization assays
- Obeys Watson-Crick base-pairing rules

A unique system for miRNA detection

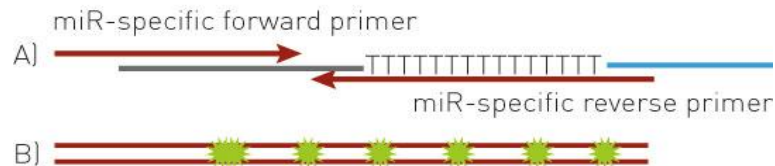
Step 1: First-strand synthesis (RT)



Polyadenylation

Reverse transcription

Step 2: Real-time PCR amplification



Two LNA™ enhanced microRNA specific primers

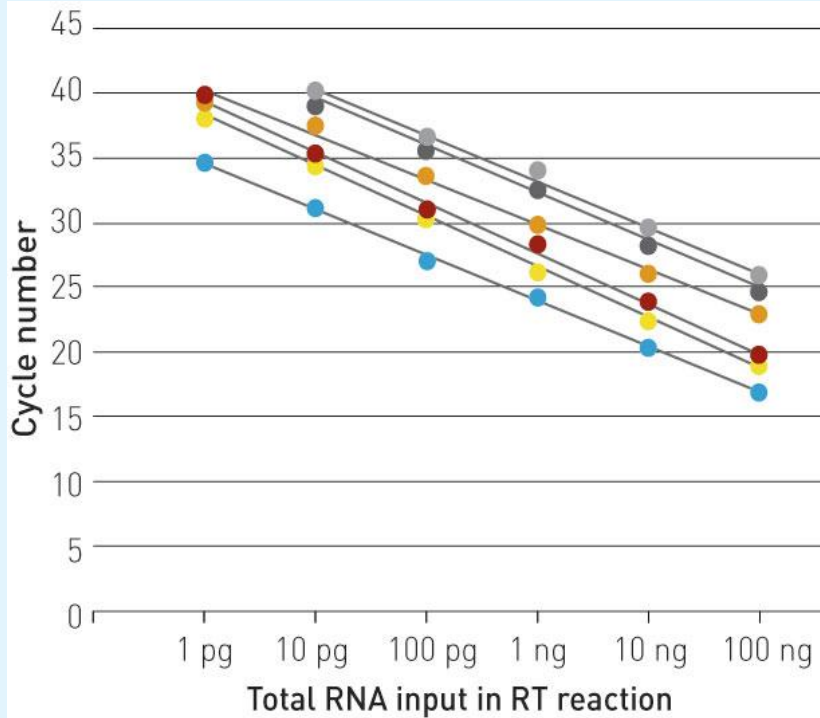
SYBR Green detection

Advantages:

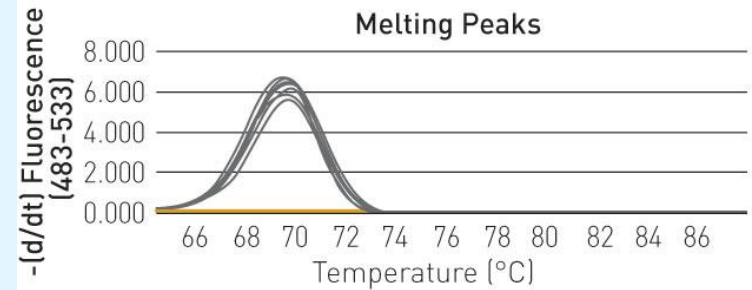
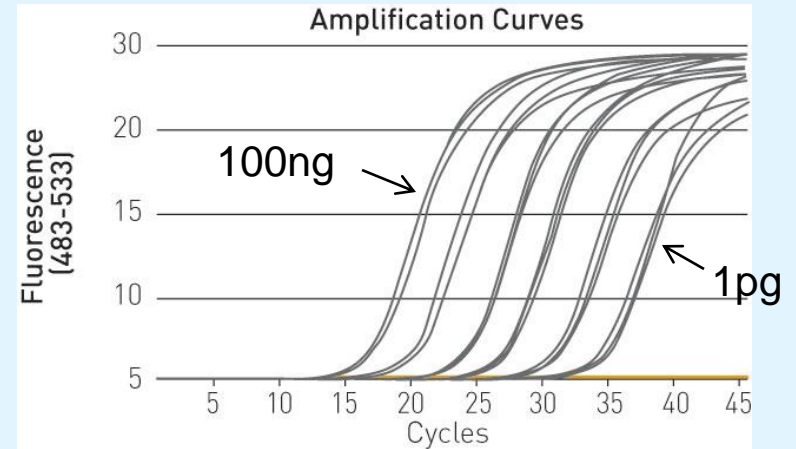
- Universal RT → ease of use
- LNA™ in two specific primers → high sensitivity and specificity

miRCURY LNA™ Universal-RT PCR system

– Specific and sensitive amplification of microRNAs



- hsa-miR-145(C)
- hsa-miR-21(C)
- hsa-let-7e(C)
- hsa-miR-145*(C)
- hsa-miR-126(H)
- hsa-miR-499-5p(H)



- hsa-miR-145
- Negative control

Single nucleotide discrimination at various positions in the microRNA target sequence

Target	Sequence	Assay	Percent detection
hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU	hsa-let-7c	100%
hsa-let-7b	UGAGGUAGUAGGUUGU G UGGUU	hsa-let-7c	0,03%
hsa-miR-99a	AACCCGUAGAUCCGAUCUUGUG	hsa-miR-99a	100%
hsa-miR-100	AACCCGUAGAUCCGA A CUUGUG	hsa-miR-99a	0,00%
hsa-miR-196b	UAGGUAGUUUCCUGUUGUUGGG	hsa-miR-196b	100%
hsa-miR-196a	UAGGUAGUUUC A UGUUGUUGGG	hsa-miR-196b	0,01%
hsa-miR-135b	UAUGGCUUUUUCAUCCUAUGUGA	hsa-miR-135b	100%
hsa-miR-135a	UAUGGCUUUU U AUCCUAUGUGA	hsa-miR-135b	0,03%
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU	hsa-let-7a	100%
hsa-let-7e	UGAGGUAG G AGGUUGUAUAGUU	hsa-let-7a	0,00%
hsa-miR-17	CAAAGUGCUUACAGUGCAGGUAG	hsa-miR-17	100%
hsa-miR-106a	A AAAGUGCUUACAGUGCAGGUAG	hsa-miR-17	2,05%

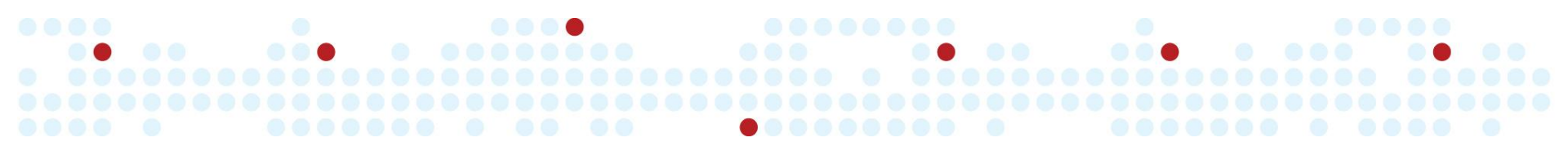
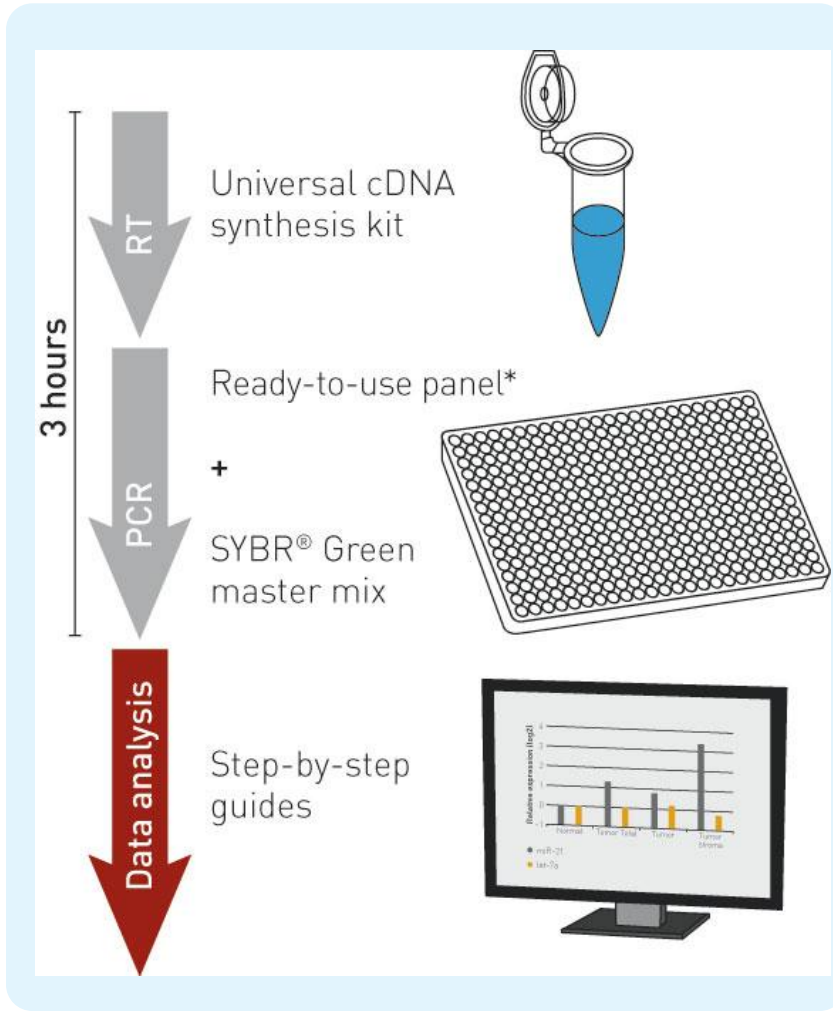
miRCURY LNA™ Universal RT PCR system

Panels

- **Human panel I+II**: 742 primer assays (384-well)
- **Mouse/Rat panel I+II**: 751 primer assays (384-well)
- **Pick-&-Mix Panels**: Select from the 742 human assays and 751 mouse&rat assays
- **Serum/plasma focus panel**: 168 microRNA assays specifically related to serum/plasma (96- or 384 well)
- **Cancer focus panel**: 88 microRNA arrays focusing on relevant human cancer microRNAs (96- or 384 well)

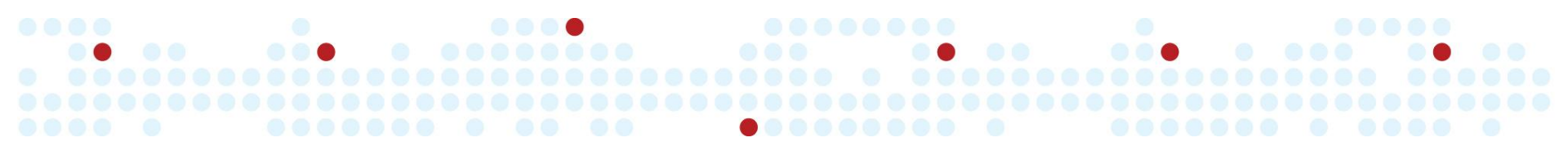
microRNA – Individual primer sets:

- 742 human primer assays
- 751 mouse/rat primer assays



Summary

- No pre-amplification is necessary – no bias introduced
- Shorter time to result
- One Universal cDNA synthesis for all microRNAs
- Better linearity – higher sensitivity
- Lower background
- Profile all miRs from only 40 ng total RNA or 70 μ L serum samples
- Melting curve analysis possible – verification of single specific amplified product
- All assays are experimentally validated and performance tested



Circulating microRNAs in blood
- promising biomarkers
Case studies



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Circulating microRNAs are stable in cell-free serum and plasma

- Major potential as biomarkers

- Serum-deprived cell lines excrete microRNAs within minutes via an energy-dependent process (Wang *et al.*, NAR, 2010).
- microRNAs are present in microparticles and exosomes, but most are found in the supernatant after ultracentrifugation, stabilized by an RNA binding protein Nucleophosmin 1.

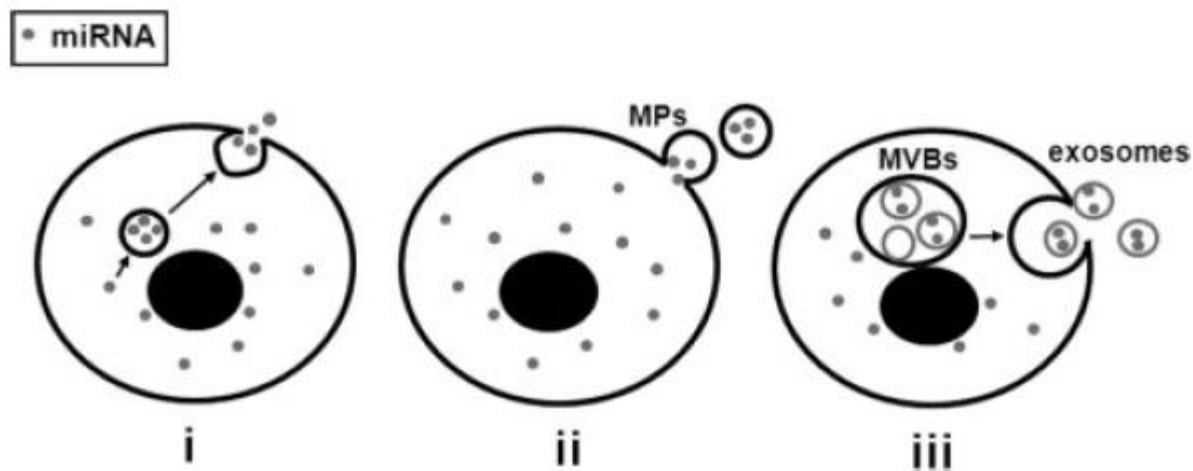
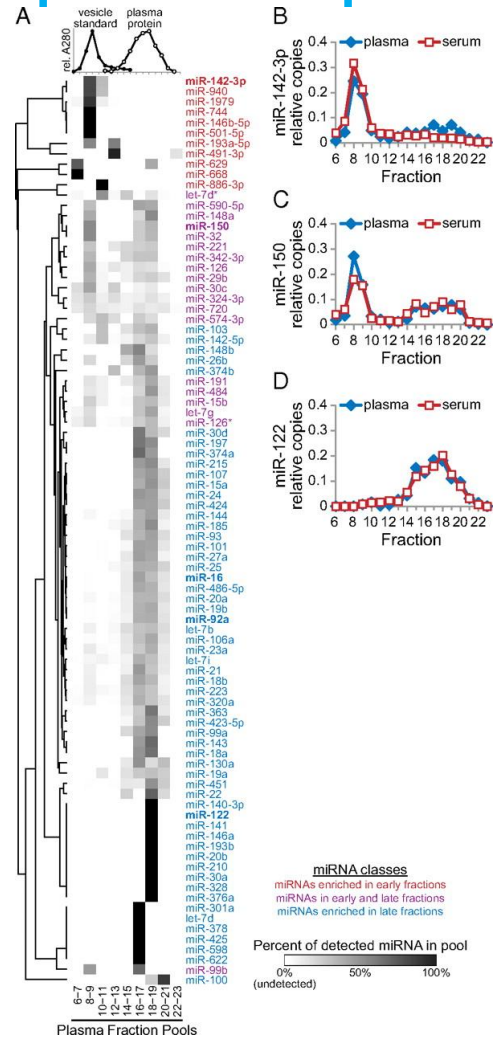


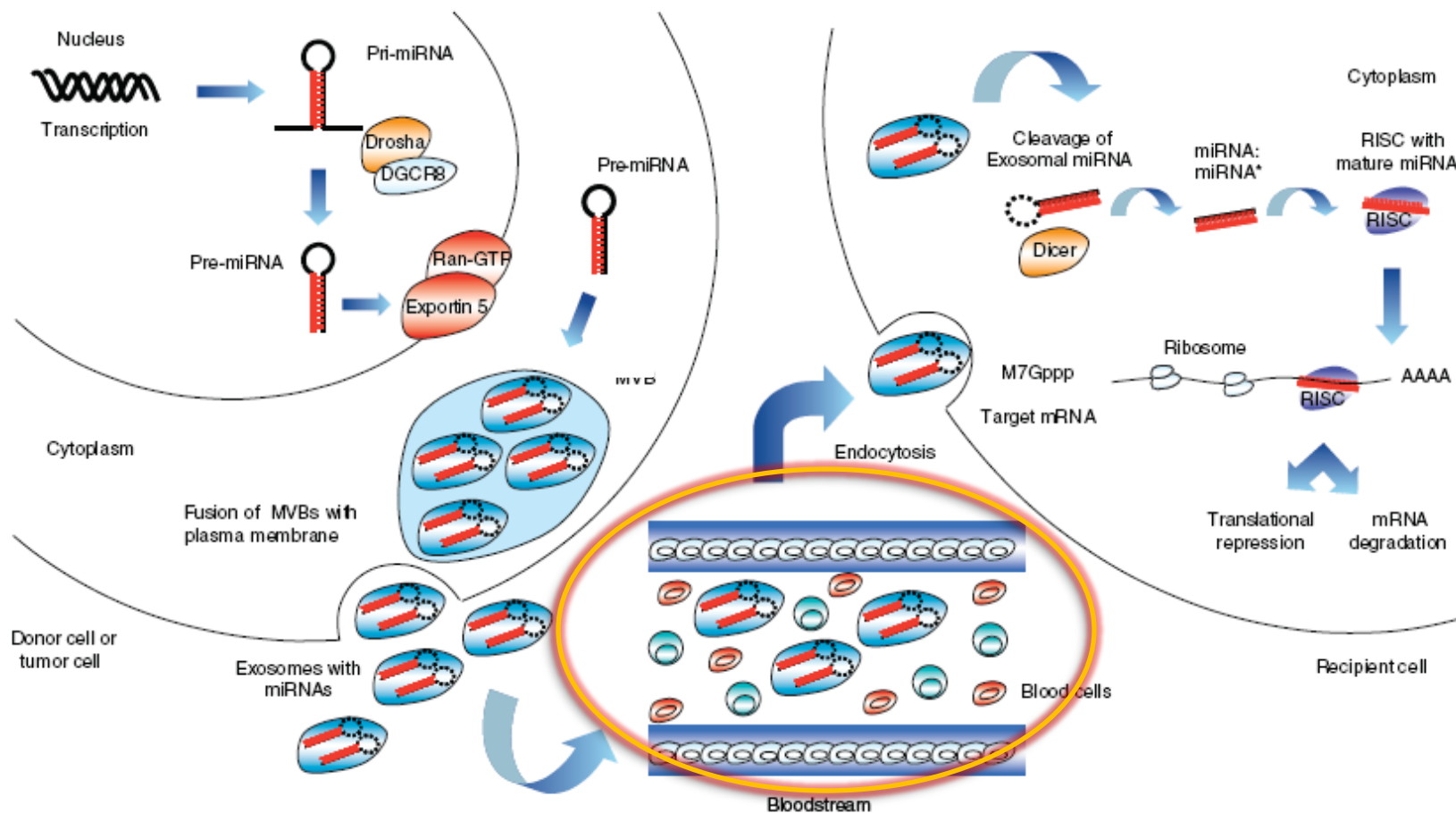
Figure 2. Schematic view of the possible sources of serum or plasma miRNAs. (i) miRNAs are secreted via exocytosis of secretory vesicles and granules; (ii) miRNAs are released via cell plasma "pinch off" microparticles (MPs); and (iii) miRNAs are released via cell-derived exosomes. MVB, multivesicular bodies.

Circulating miRNAs are predominantly in fractions consistent with ribonucleoprotein complexes.

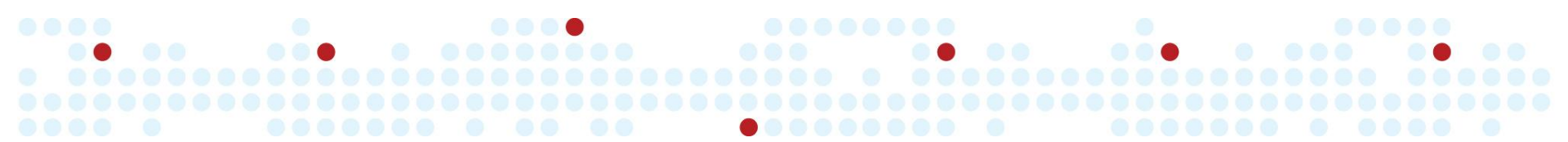
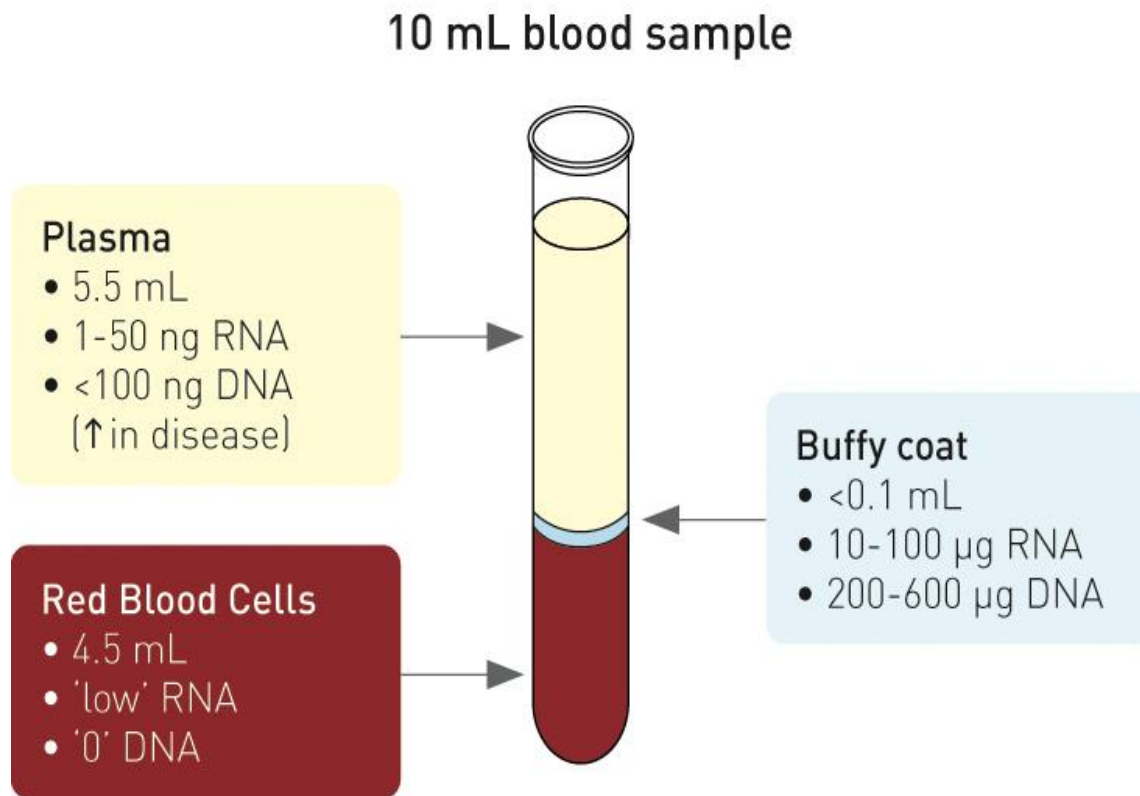


Arroyo J D et al. PNAS 2011;108:5003-5008

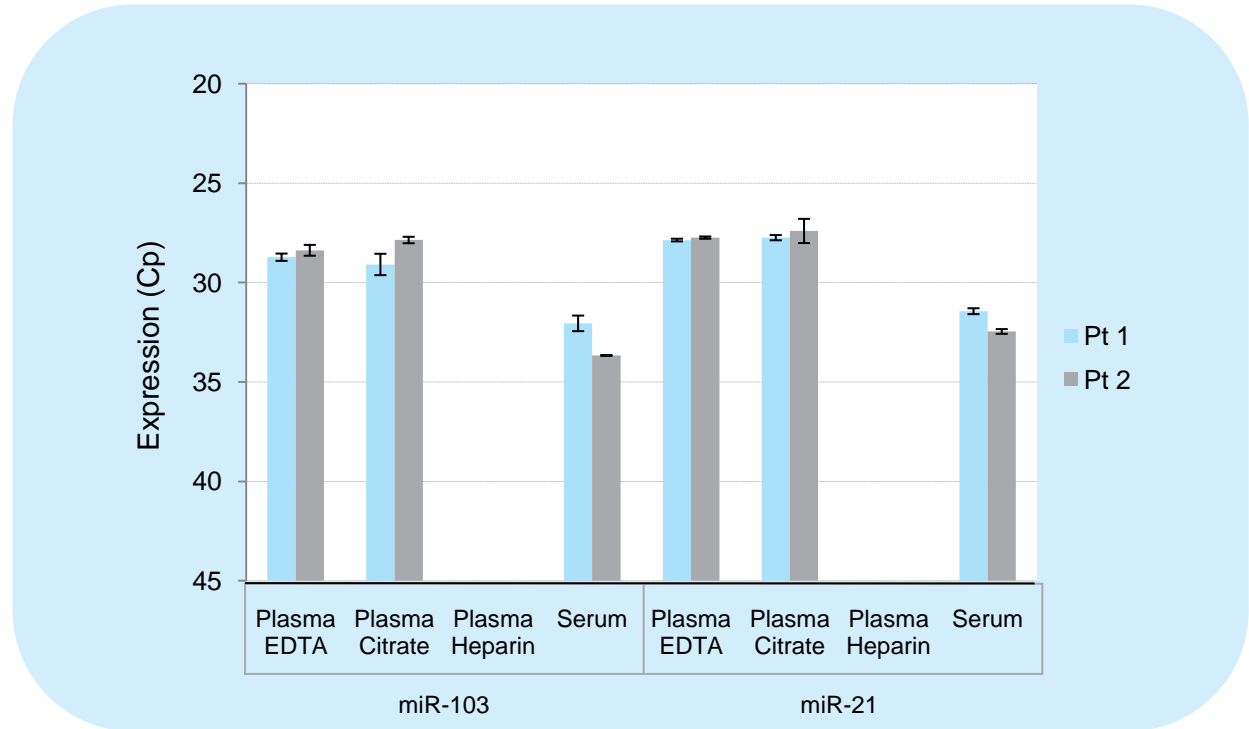
Circulating miRNA may be a parallel to the endocrine system important for transmission of signals in the body



Limited amount of RNA in plasma samples



Exiqon- Blood collection tubes and subsequent effect on miRNA QPCR

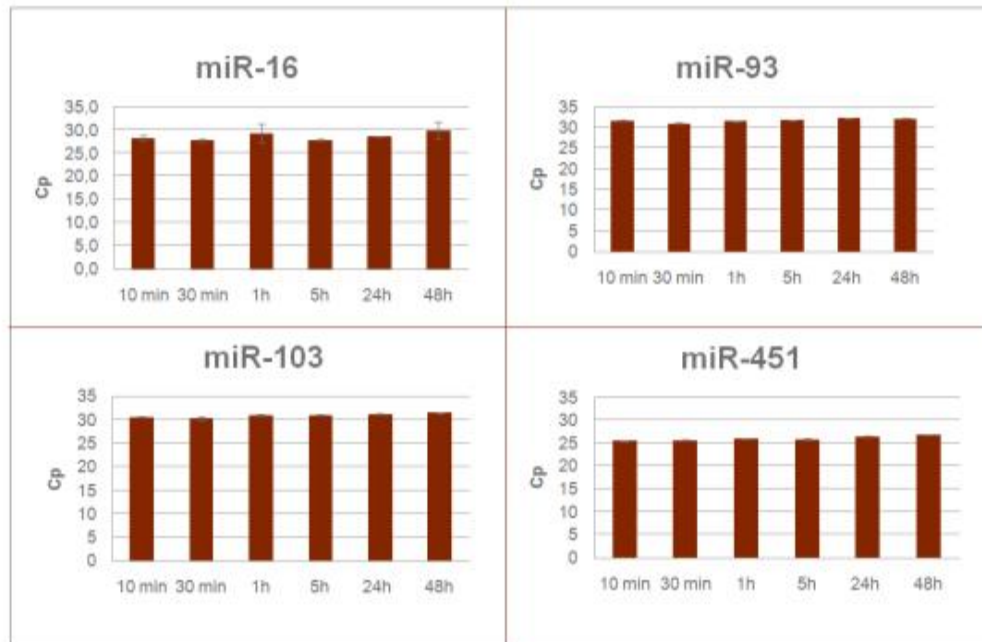


Circulating microRNAs are stable in cell-free serum and plasma - microRNA profile insensitive to sample handling

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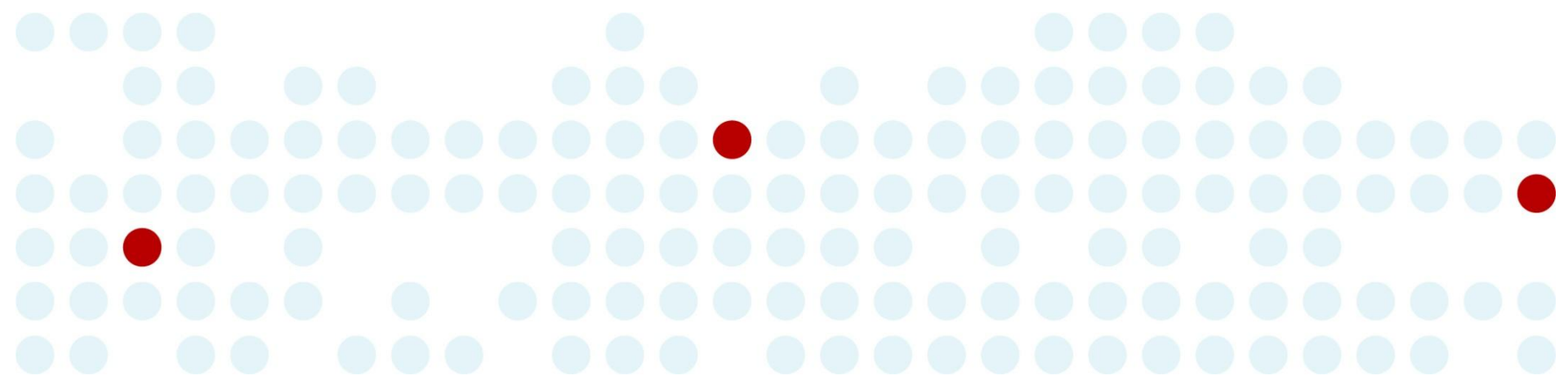
FACULTY OF LIFE SCIENCES

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



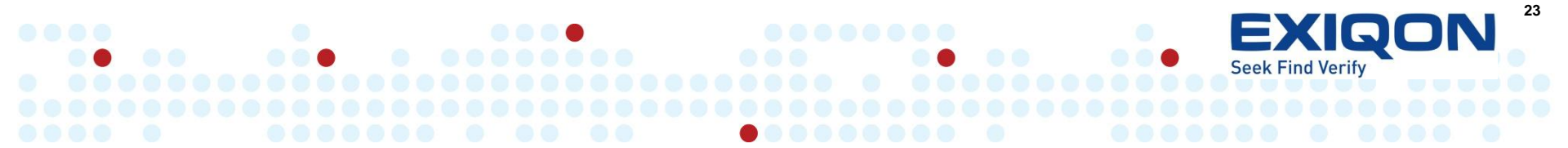
Place, date, unit, occasion etc.
Slide 1





Case studies –

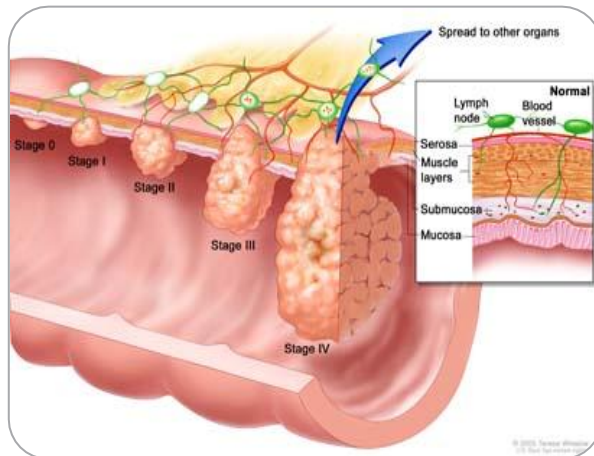
microRNA biomarkers from human patient
blood serum/plasma



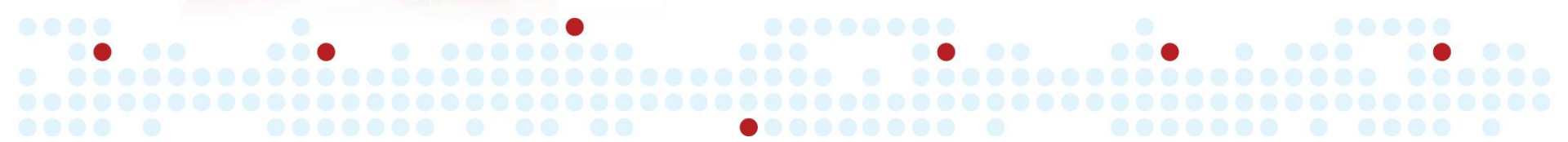
Development of miRNA Early Detection Test of CRC in blood plasma

Colorectal cancer

- Estimated new cases in 2009 in the USA:
 - Colon: 106,100
 - Rectum: 40,870
- Estimated deaths in 2009 in the USA:
 - Combined: 49,920
- **Early diagnosis results in operable cancer with much improved prognosis**



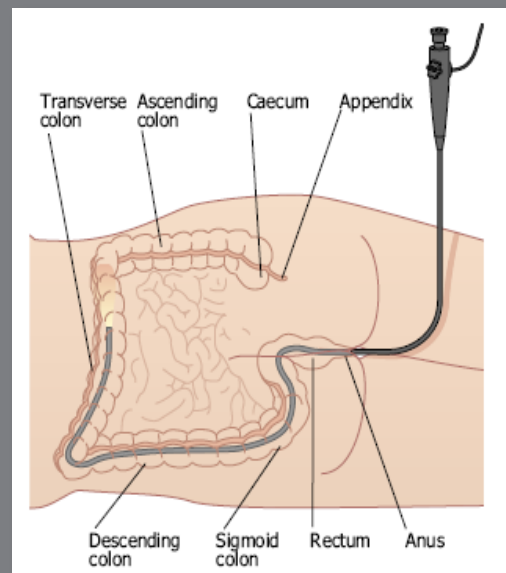
Stage	5 yr relative survival	Treatment
0-I	93%	Surgery
II	80%	Surgery/discretionary adjuvant chemotherapy
III	58%	Surgery/adjuvant chemotherapy
IV	6.9%	Chemotherapy



Development of miRNA Early Detection Test of CRC in blood plasma

USA Colorectal cancer (CRC) screening guidelines

- For individuals between 50 and 75:
 - Colonoscopy every 10 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**



Work flow compatible with standard clinical procedures

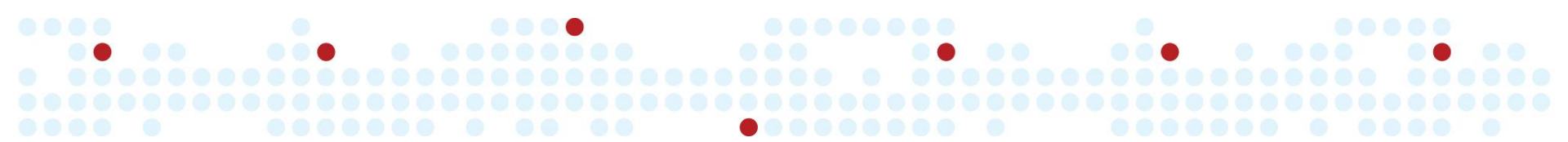
Total lab time = 5 hours				
	time = 1 hour	time = 1.5 hours	time = 2 hours	time = 0.5 hours
Plasma sample	RNA isolation	RT reaction	PCR reaction	Data QC and analysis.
<ul style="list-style-type: none"> Standard Hospital procedures 	<ul style="list-style-type: none"> RNA isolation 200ul plasma required Isolation of total RNA for from sample (no bias) 	<ul style="list-style-type: none"> Standard RT reaction. Robust and reproducible 	<ul style="list-style-type: none"> PCR with LNA specific primers for detection of miRNAs 	<ul style="list-style-type: none"> Quality control Data analysis and results Diagnostic interpretation



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



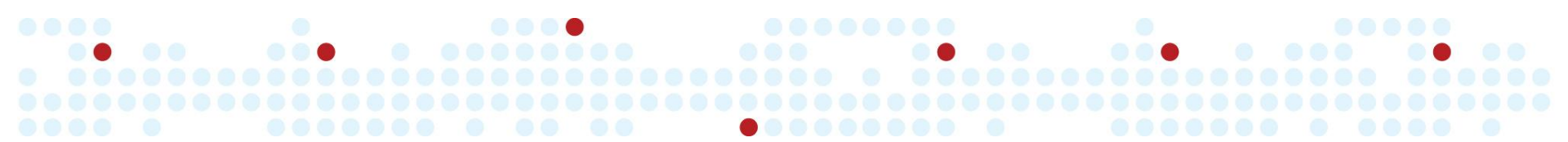
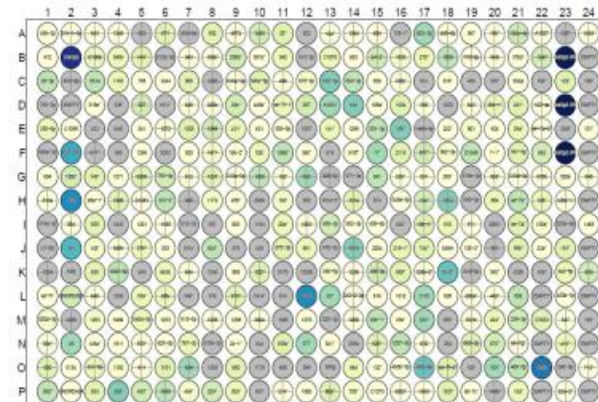
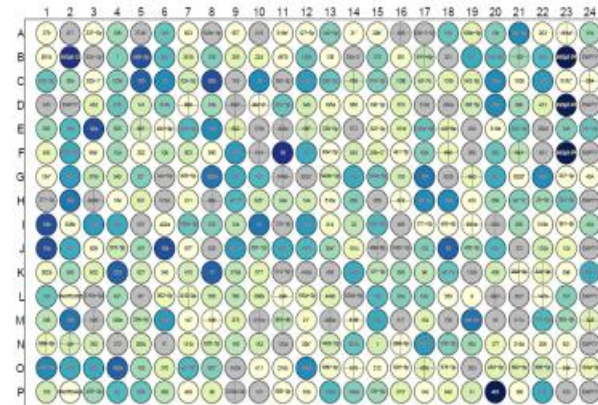
- FDA approved technology
- Conventional qPCR
- No black box algorithms
- Automatable



Development of miRNA Early Detection Test of CRC in blood plasma

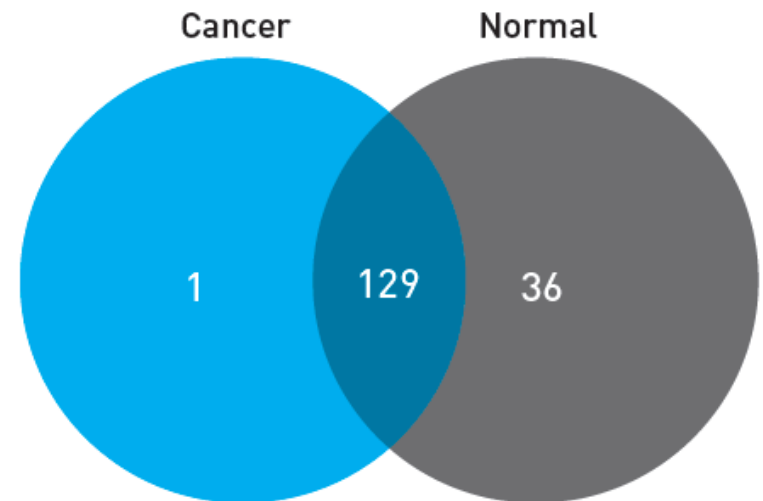
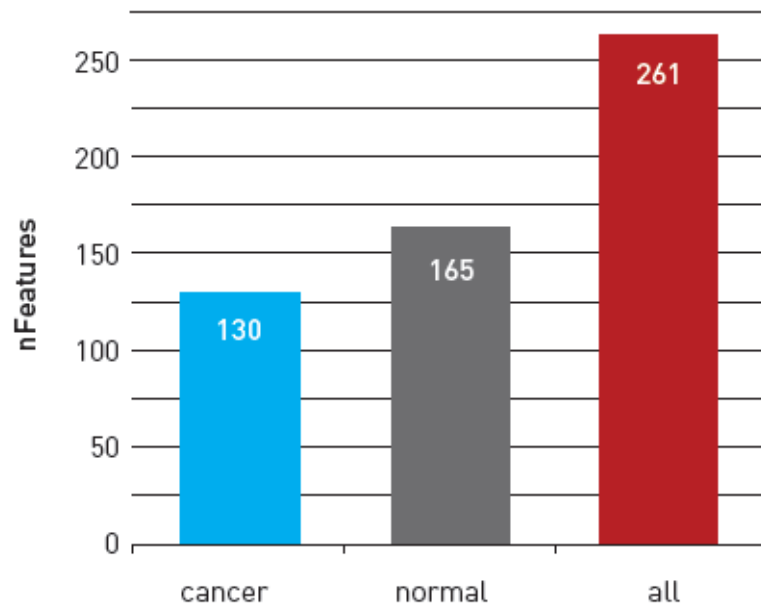
Biomarker discovery from blood plasma

- 50 stage II colorectal cancer patients
- 50 age- and sex-matched colonoscopy-negative controls
- **Plasma** samples (pre-endoscopy), 200 μ L
- Whole genome microRNA profiling using LNA Universal RT microRNA PCR Panels
- List of miRNAs expressed in plasma



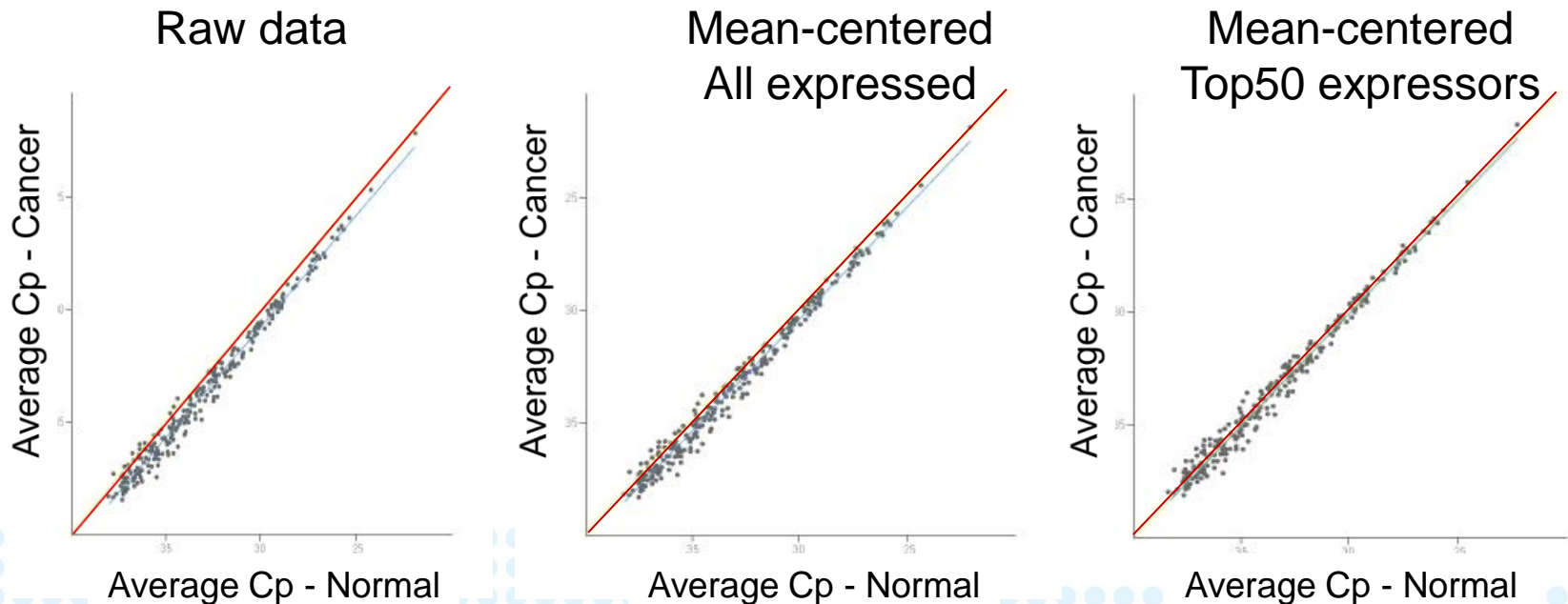
Call rate for microRNAs in archival plasma samples from CRC patients and controls

- 261 microRNAs detected in at least one sample
- 166 microRNAs detected in >90% per sample group



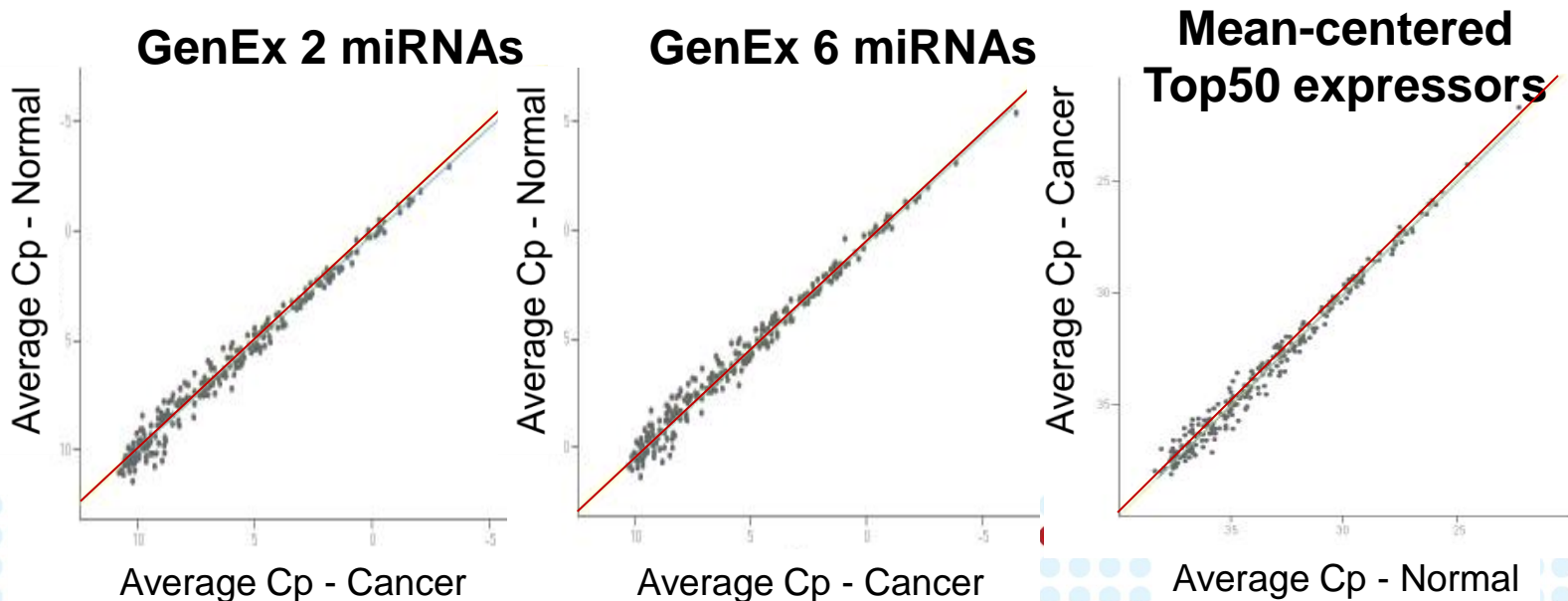
Global data normalization is best method to normalize this large plasma dataset

- **Plasma** samples (pre-endoscopy)
- Whole genome microRNA profiling: LNA Universal RT microRNA PCR Panels
- Mean-centered Top50 expressors performs best for this dataset



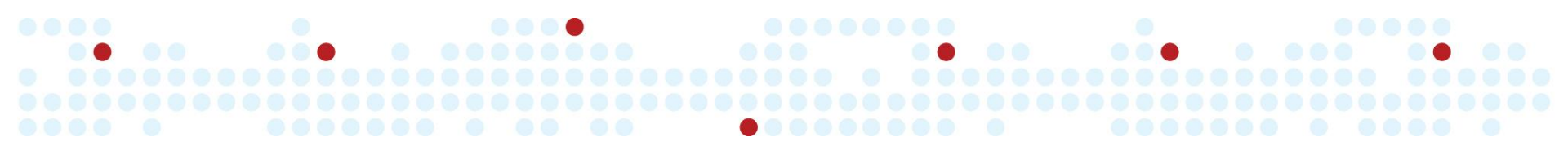
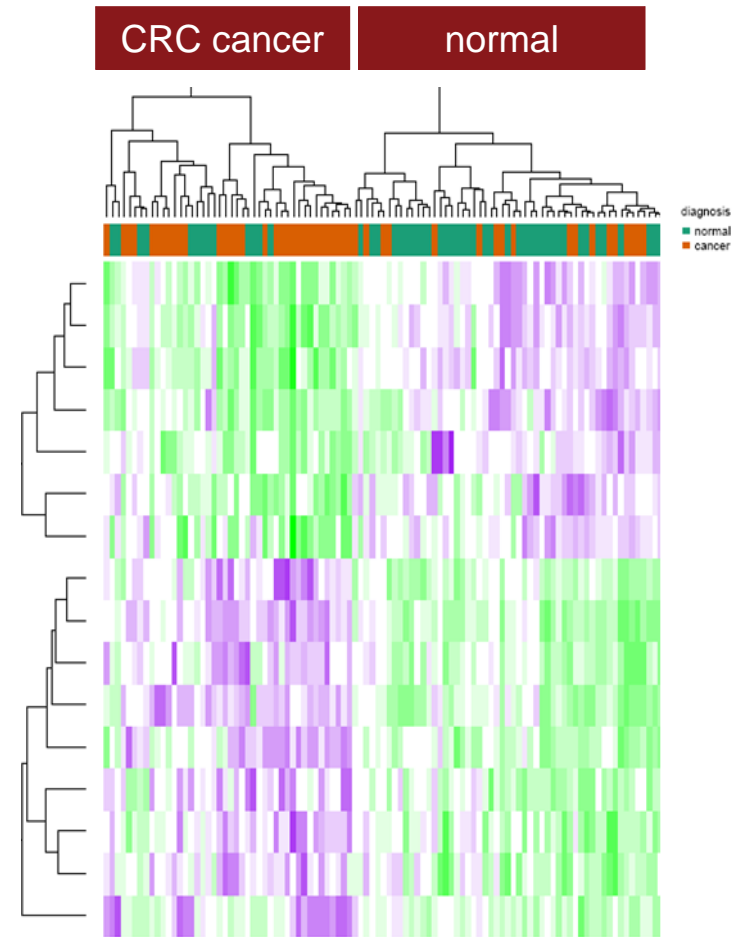
Reference gene normalization using GenEx performs well

- **Plasma** samples (pre-endoscopy)
- Whole genome microRNA profiling: LNA Universal RT microRNA PCR Panels
- Discovery of reference miRNAs that follow global mean using GenEx software
- Normalization using 2 or 6 reference microRNAs is acceptable

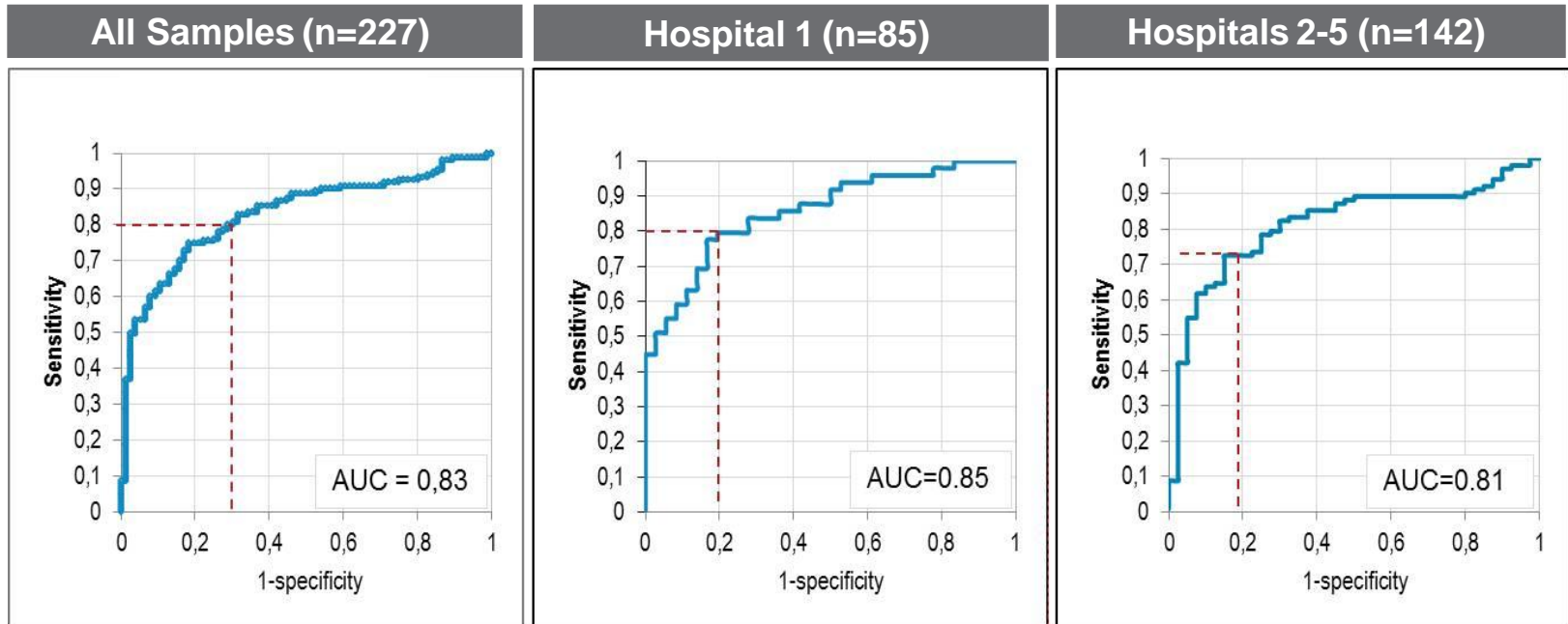


Pre-screen: Profile differs in plasma from CRC patients and healthy controls

- 50 stage II colorectal cancer patients and 50 age- and sex-matched colonoscopy negative controls
- Plasma samples (pre-endoscopy)
- Supervised approach on 730 miRNAs. Lasso-based feature selection and simple classifier. 10-fold X-validation
- Screening defined 378 candidate miRNAs present in plasma

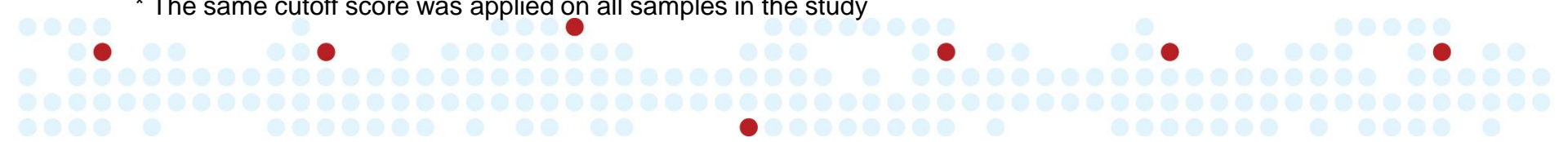


Focused panel of miRNAs in plasma may be used as biomarker for CRC



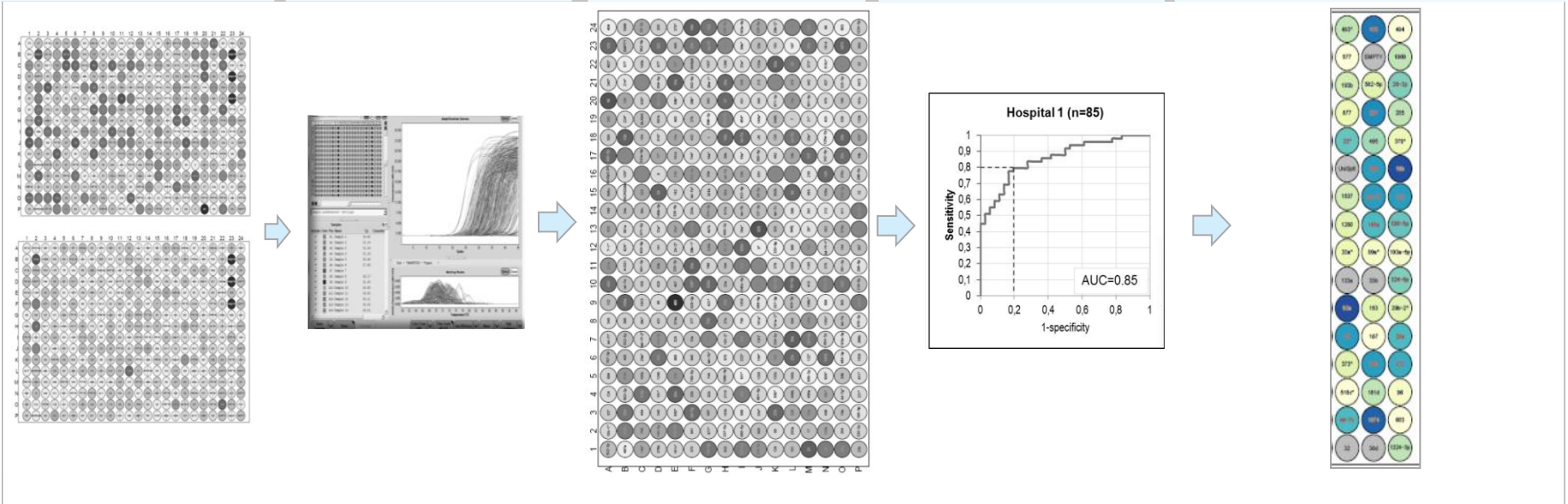
	All Samples	Hospital 1	Hospitals 2-5
Sensitivity *	75%	80%	73%
Specificity *	80%	78%	82%
(n) Cancer	151	49	102
(n) Control	76	36	40

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



Development of miRNA Early Detection Test of CRC in blood plasma

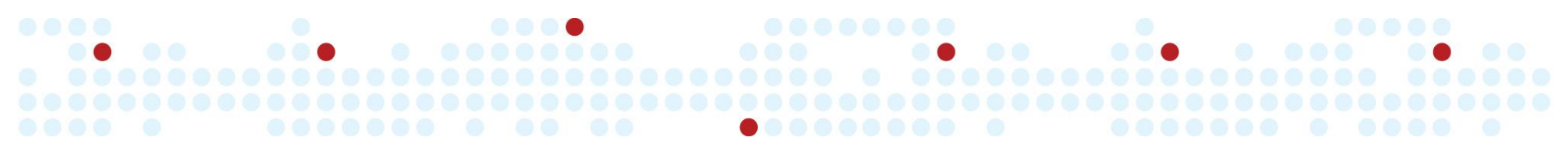
DISCOVERY PHASE				VALIDATION PHASE
Genome wide screening	Normalization, QC, processing	Candidate miRNA discovery screen.	Bioinformatics, data analysis,	Validation Set miRNA signature .
<ul style="list-style-type: none"> 50 controls 50 CRC patients 730 miRNA screen 	<ul style="list-style-type: none"> Multiple QC check Data flagging Normalization 	<ul style="list-style-type: none"> 76 controls 151 CRC patients 378 custom miRNAs screened Multiple controls 	<ul style="list-style-type: none"> Data analysis Quality control ROC curve miRNA selection 	<ul style="list-style-type: none"> 3000 patients (2011) Defined miRNA signature Multiple controls



Development of miRNA Early Detection Test of CRC in blood plasma

Conclusions:

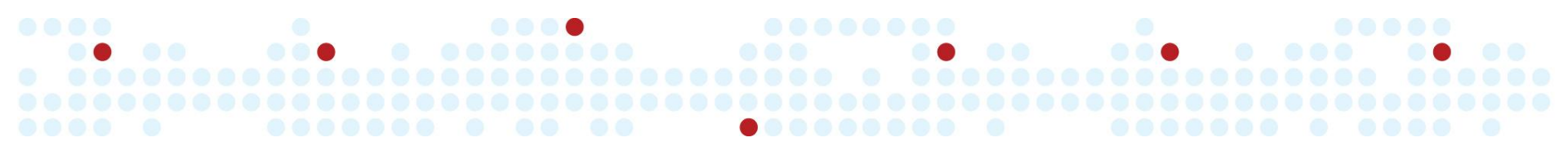
- Robust and reliable platform for detection of miRNA in plasma/serum
- miRNAs fulfill the requirements for being a clinical applicable biomarker
- miRNA profile in plasma/serum may be applied for early detection of CRC
- miRNA profile obtained from 200 μ l blood in 5 hours
- Major validation study (3,000 individuals) to be completed late 2011



Screen for serum miRNAs in responders to experimental drug

The experiment

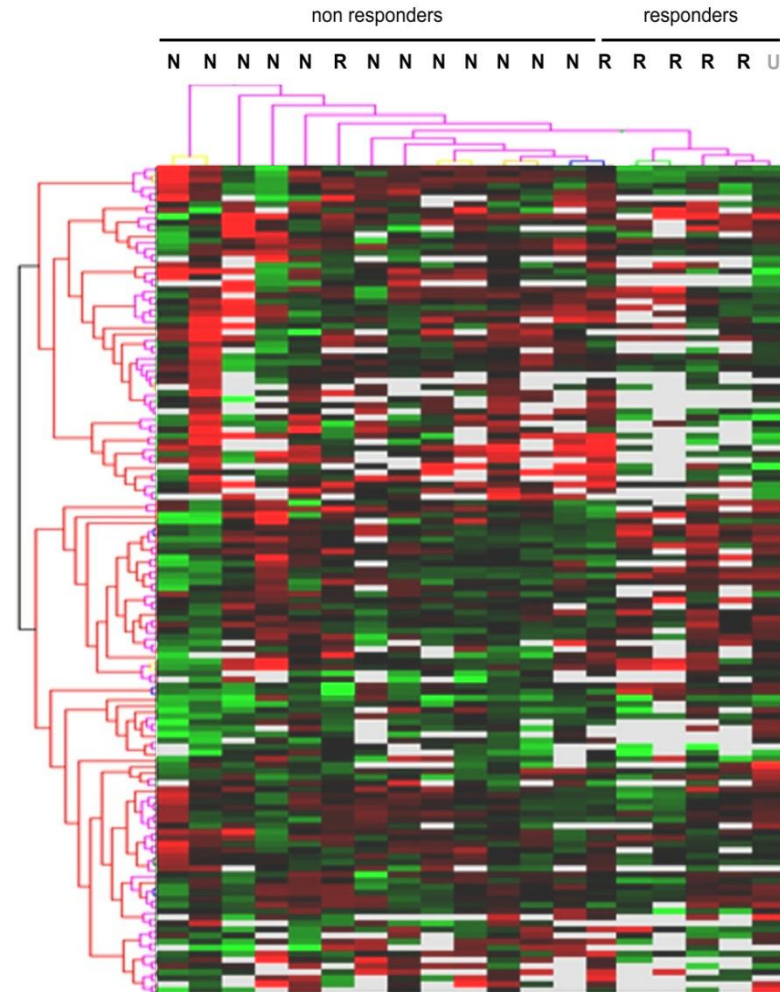
- 29 patients treated with experimental drug
 - 1 sample pre-treatment
 - 1 sample post-treatment
- **Serum** samples
- Whole genome microRNA profiling using LNA Universal RT microRNA PCR Panels
- 119 miRNAs detected in >80% of samples post QC



Screen for serum miRNAs regulated in responders to experimental drug

Conclusions










- Unsupervised clustering using all miRNAs generally clusters samples from same patient (data not shown)
- Unsupervised clustering of samples based on change between visits separates responders (R) from non-responders (N). (U is unknown response)

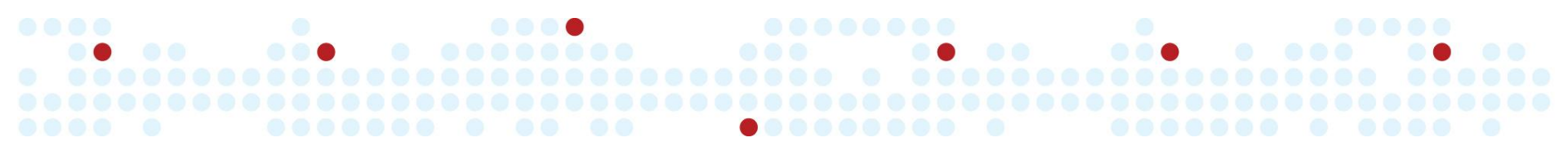


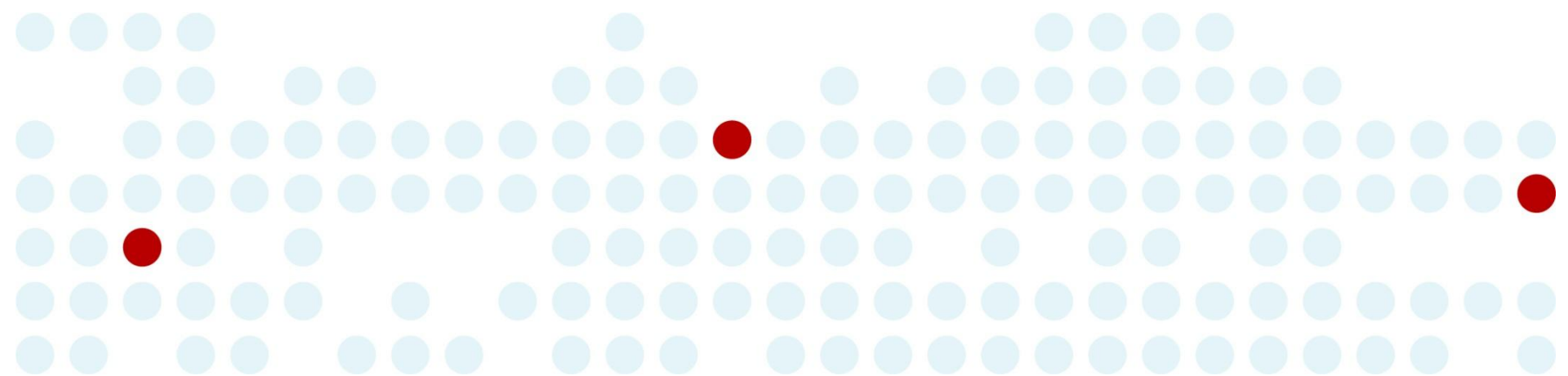
Summary

- Circulating MicroRNAs in blood and are promising biomarkers
- microRNAs in serum/plasma samples are very stable
- Serum/plasma microRNA profiles are affected by various diseases – cancer in particular.
- **miRCURY LNA™ Universal RT microRNA PCR system** enables highly sensitive and accurate identification of biomarkers in blood serum/plasma samples.
- *This will change the way we predict and diagnose diseases in the future!*

Exiqon: Established one-stop shop for miRNA research products

Process	 Isolation	 Expression Analysis	 Localisation	 Functional Analysis	
Product	 <p>miRCURY™ sample isolation system</p>	 <p>miRCURY LNA™ miRNA Array System</p>	 <p>miRCURY LNA™ miRNA PCR System</p>	 <p>miRCURY LNA™ miRNA Detection Probes</p>	 <p>miRCURY LNA™ miRNA Knockdown/ Inhibition</p>
Service	✓	✓	✓	✓	





Thank you for your attention

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