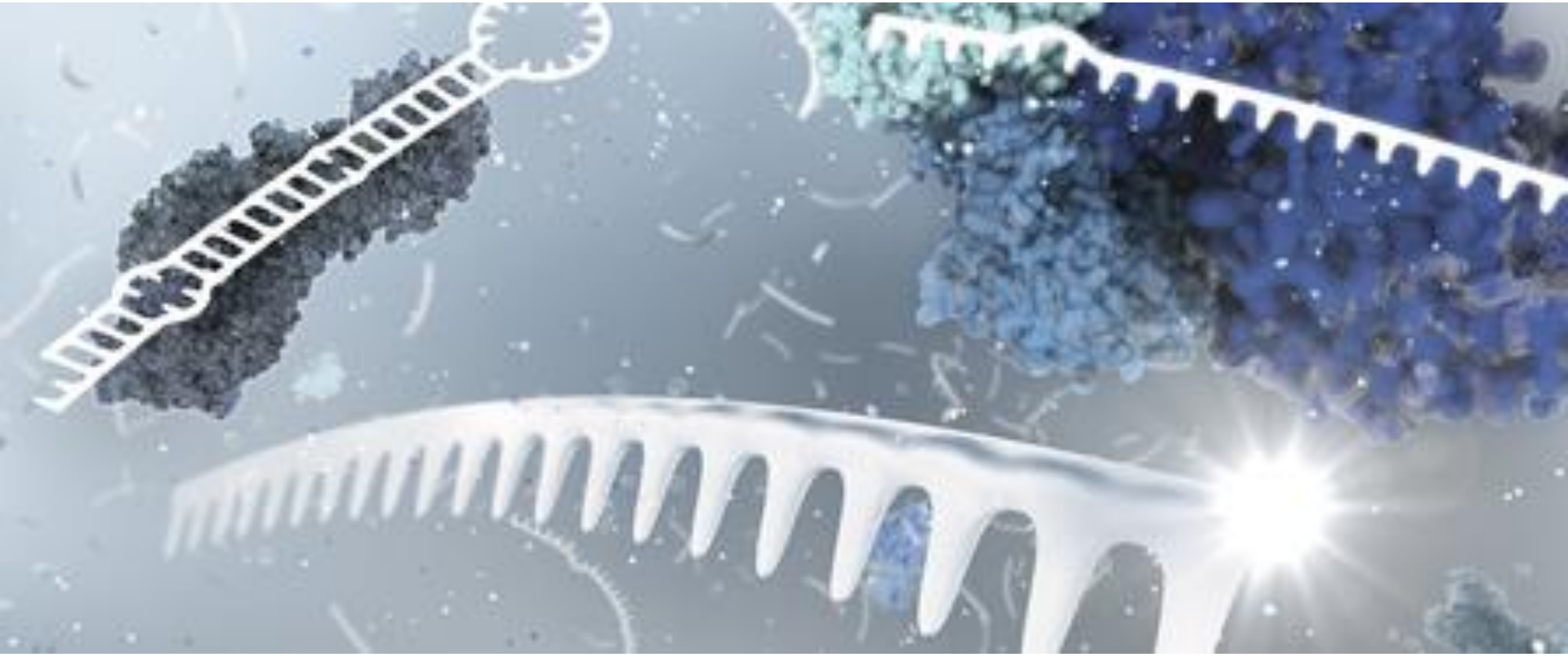
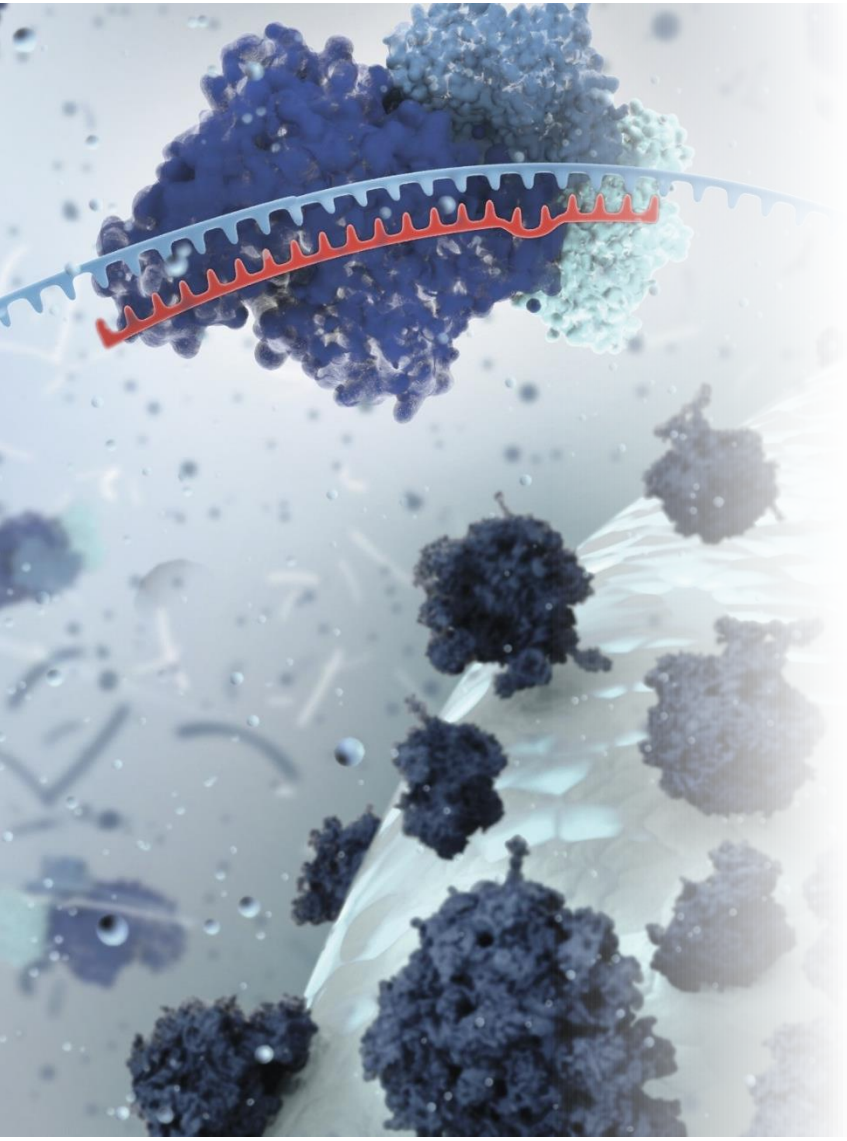
# miRNA Research Using Advanced qPCR Technology

## Successfully Detect miRNAs Using qPCR with LNA Technology



Dr. Francesca Di Pasquale, Associate Director R&D

# Two-part webinar series on circulating RNA biomarkers



## Part 1: Advancing miRNA Research – Best Practices for Your Experiments

- Best practices and tools to consider at each step of your miRNA experiment

## Part 2: Successfully Detect miRNAs Using qPCR with LNA Technology

- Successfully detect miRNAs qPCR with LNA (Locked Nucleic Acids) Technology

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# Agenda

1. miRNAs and their dysregulation
2. miRNA detection methods
3. LNA technology
4. The miRCURY LNA miRNA PCR Detection System
5. Working with biofluids
6. Outlook

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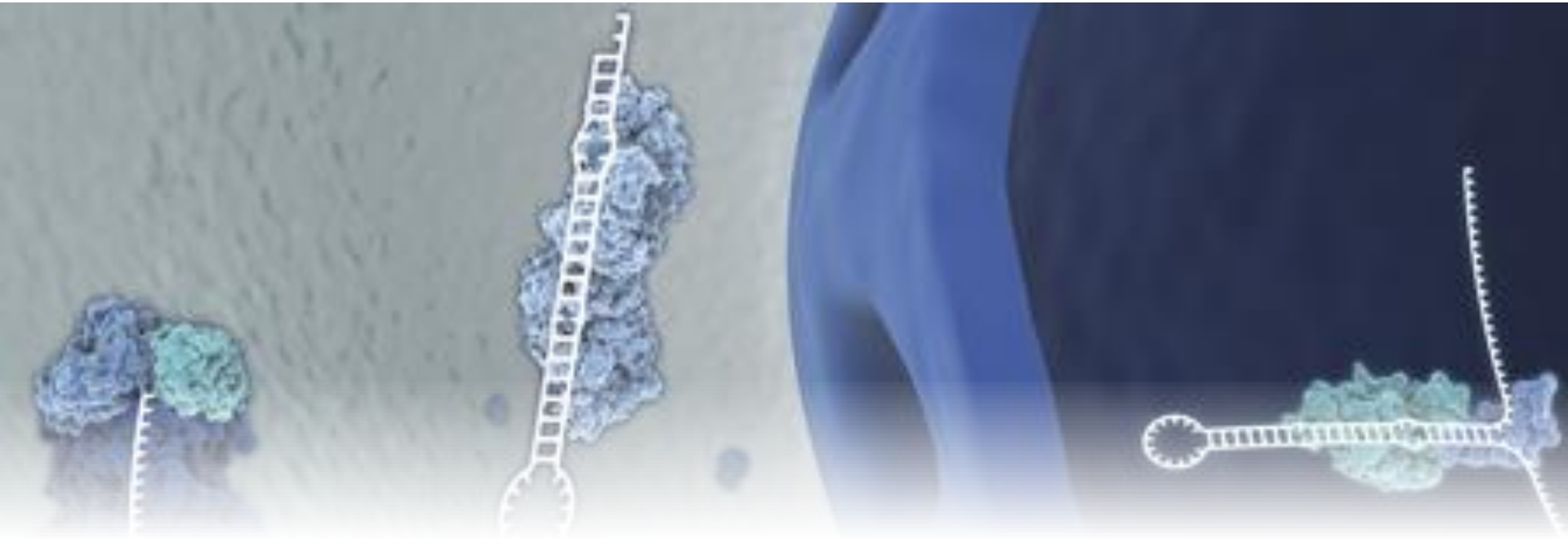
# miRNAs and biogenesis



## Mature (miRNAs)

- miRNAs are naturally occurring, ca. 22-nt, noncoding RNAs that mediate post-transcriptional gene regulation
- Gene regulation is highly specific
- miRNAs usually complementary to a 3' UTR site
- Bind to and repress specific mRNA targets

## Biogenesis

- Transcribed by RNA Polymerase II as a long primary transcript (pri-miRNAs)
- In the nucleus, pri-miRNAs are processed to hairpin-like pre-miRNAs by RNAse III-like enzyme Drosha, then exported to the cytosol
- In the cytosol RNAse III-like Dicer processes these precursors to mature miRNAs
- These miRNAs are incorporated in RISC

# Mechanism and dysregulation

## miRNAs work in the RIS complex

- Binds miRNAs that act as a template to recognize and cleave a complementary mRNA
- miRNAs with imperfect base pairing to the target mRNA lead to translational repression and/or mRNA degradation

## Dysregulation of miRNA expression is a cause or indicator of several disease processes

- There are hundreds of identified miRNAs for many model organisms
- i.e. role of hsa-miR-1 in cardiac hypertrophy http://www.mir2disease.org/

Source: Danish Sayed et. al. Circulation Research. 2007;100:416-424, February 16, 2007

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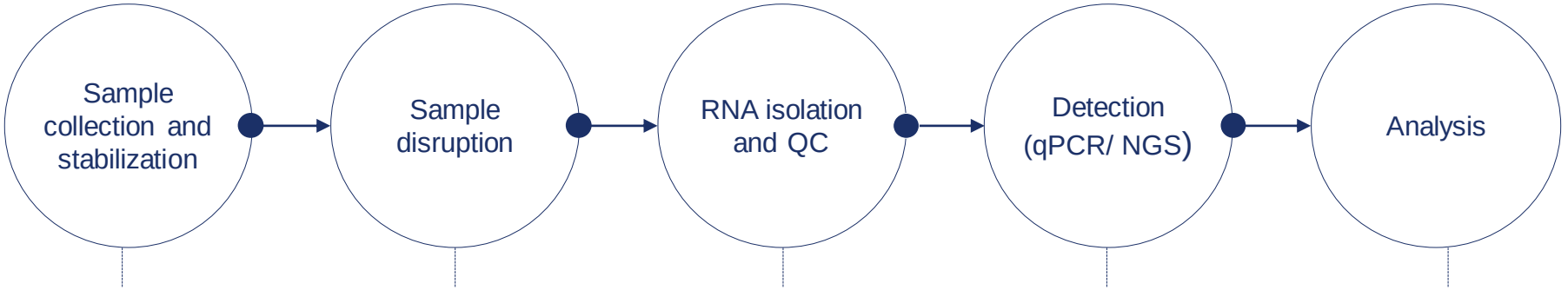
# Common challenges when working with RNA or miRNA




- Time between collection and stabilization/lysis
  - ▶ Changes in RNA transcript profile/ degradation
  - ▶ Sample degradation
- Lysis condition (tissue/cells)
- Efficiency of lysis
- Sample size
  - ▶ Sample degradation
  - ▶ Contamination
- RNA quantification
- gDNA contamination
- RNA integrity
  - ▶ Over/ underestimated yields
  - ▶ Low integrity
- Primer design
- Efficiency of library construction
  - ▶ Biased target amplification
  - ▶ Biased library preparation
  - ▶ Detection limit for low abundant transcripts
- Accurate data analysis and interpretation
  - ▶ Low abundant transcripts
  - ▶ Accurate estimation of expression levels /normalization

Each of these steps has its own challenges

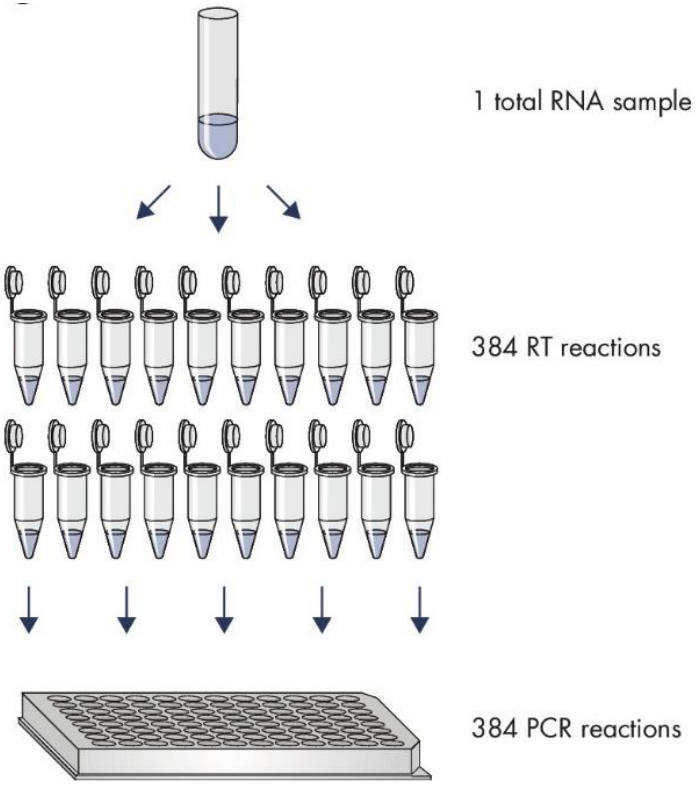
## Advantages and disadvantages

### miRNA detection

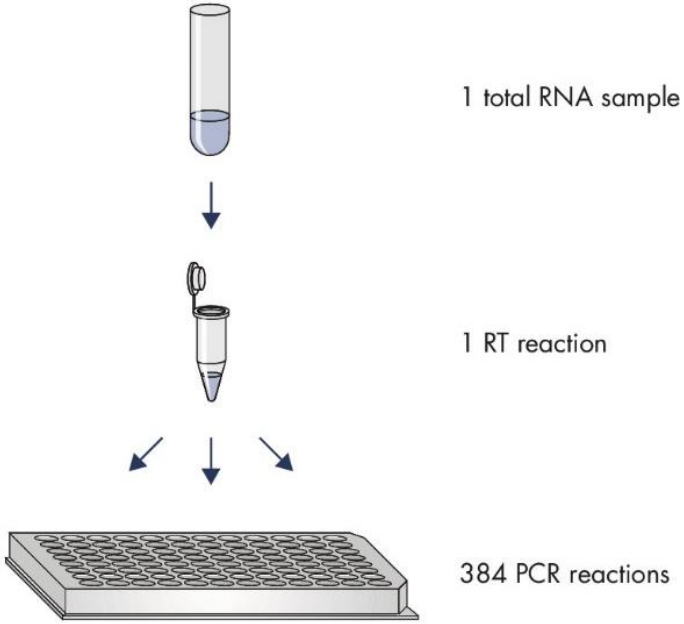
| qPCR | NGS | NGS + qPCR |
|---|---|---|
| 1 Screening possible, mostly hypothesis-driven | 1 Explorative screening | 1 Screening + validation |
| 2 Fast method | 2 “Big picture” | 2 Independent method for confirmation |
| 3 Cost-saving | 3 Work and cost-intensive | 3 Further studies with a small group of targets |

## Advantage of the universal RT reaction

A) miRNA-specific RT reaction

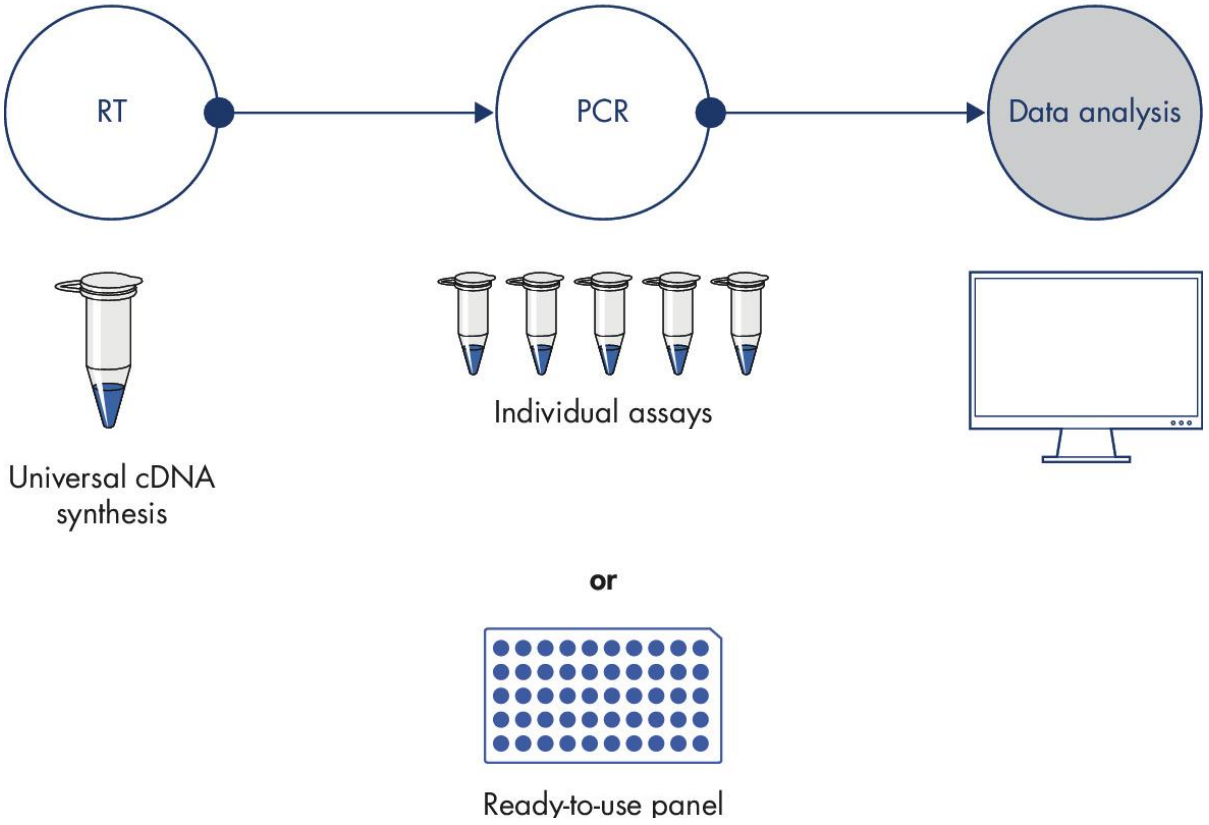



B) Universal RT reaction




- Only one RT reaction obtaining the cDNA for all miRNAs contained in one sample

## Typical workflow for the detection of miRNAs using qPCR




- 3 hour workflow – fast results using the miRCURY system

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EXIQON

Now a QIAGEN company

QIAGEN

## Exiqon product portfolio now by QIAGEN

Since Oct 1, 2017 Exiqon products are on the QIAGEN.com webshop

- Exiqon products are now in GeneGlobe
- Products remain vastly unchanged
- QIAGEN benefits from the proprietary LNA technology

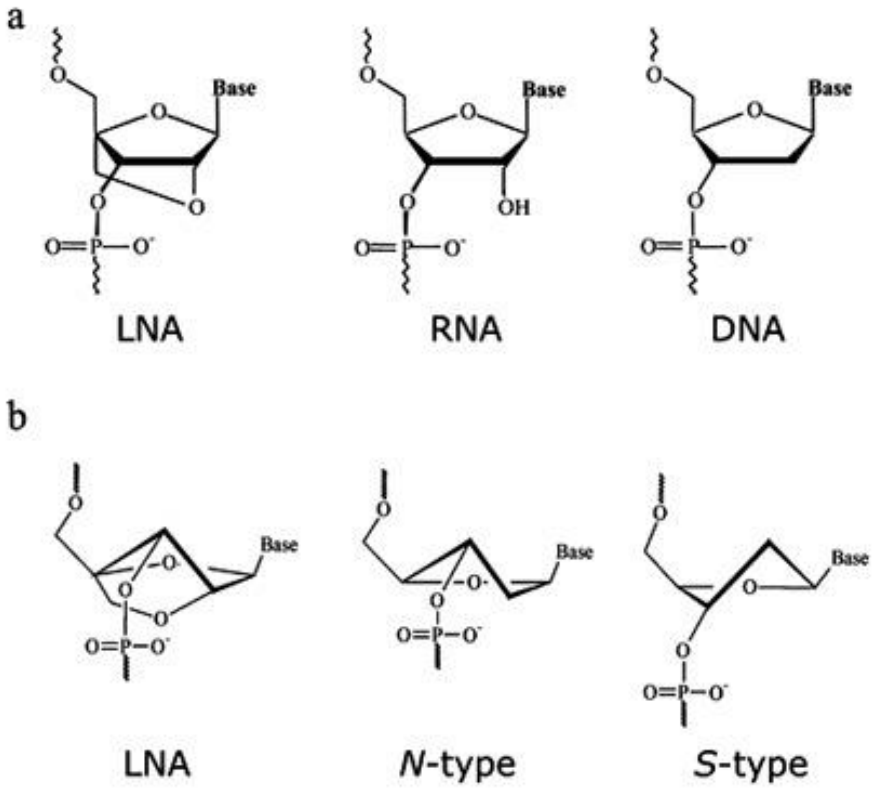
## Improved through QIAGEN quality

The qPCR system was improved with the latest versions of QIAGEN´s superior enzymes

- Offer a system that is produced by QIAGEN
- According ISO 9001 (reagents also ISO 13485 and cGMP certified)
- Set our own quality and QC standards

## Bicyclic RNA mimic with the sugar ring locked in the 3'-endo conformation

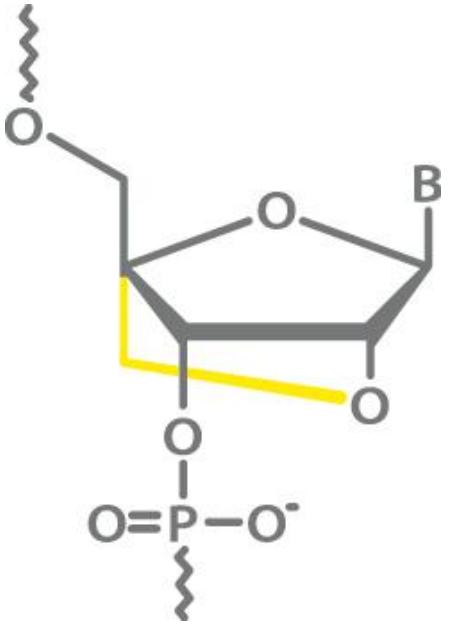
- Reducing its conformational flexibility
- Increasing the local organization of the phosphate backbone




Source: Silahtaroglu, Pfundheller, Koshkin, Tommerupa, Kauppinen Cytogenetic and Genome Research 107(1-2):32-7 · February 2004

## LNA technology improves binding affinity

- Obeys Watson-Crick base-pairing rules
- Stable A-helix with good base-stacking




- Increased $T_m$ (2–8 ºC per base)
- Improved mismatch discrimination
- High sensitivity and specificity in probes and assays
- Increase oligo stability and potency in cells

*Bondensgaard et al., Chem. Eur. J. 2000*
*Petersen et al., J. Am. Chem. Soc. 2002*

Making oligos with unprecedented characteristics

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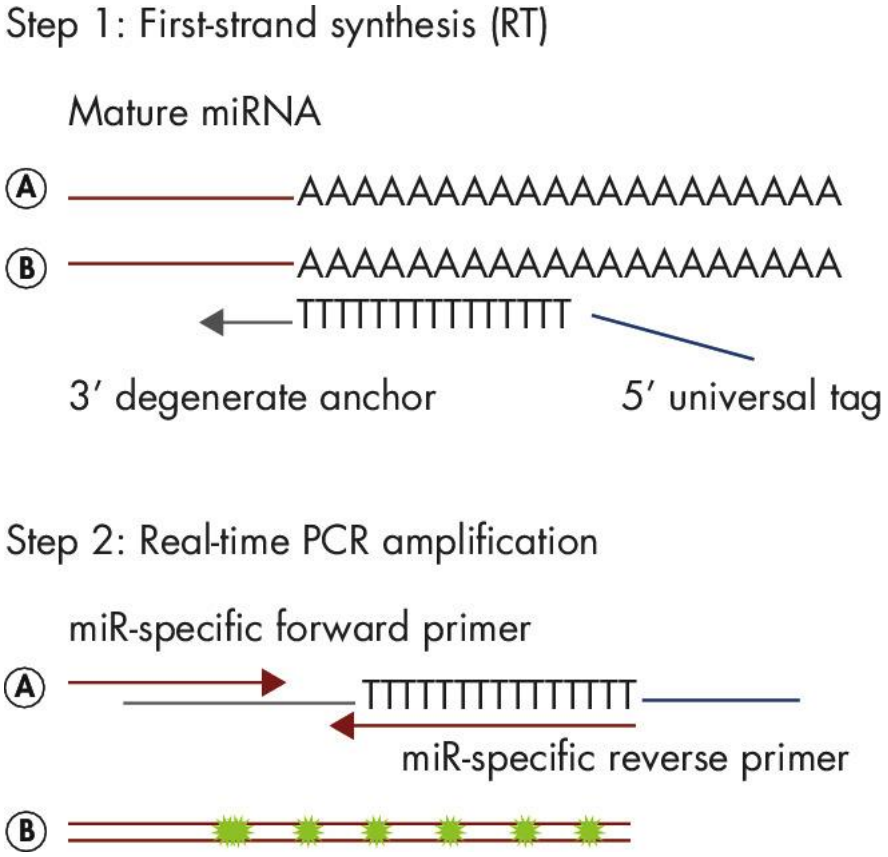
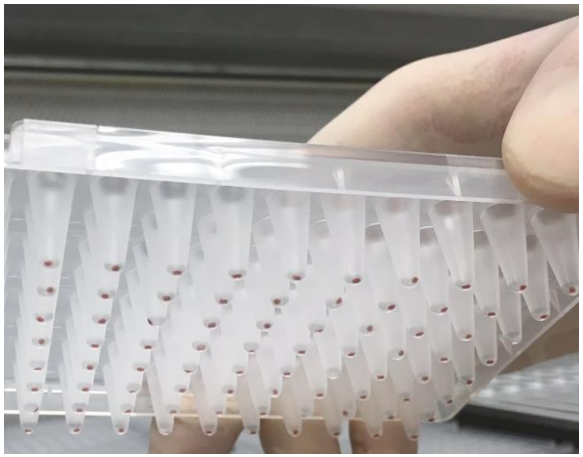
## miRNA qPCR detection system using LNAs




- High specificity due to two miRNA specific primers

## Already know the miRCURY system? … There are some changes

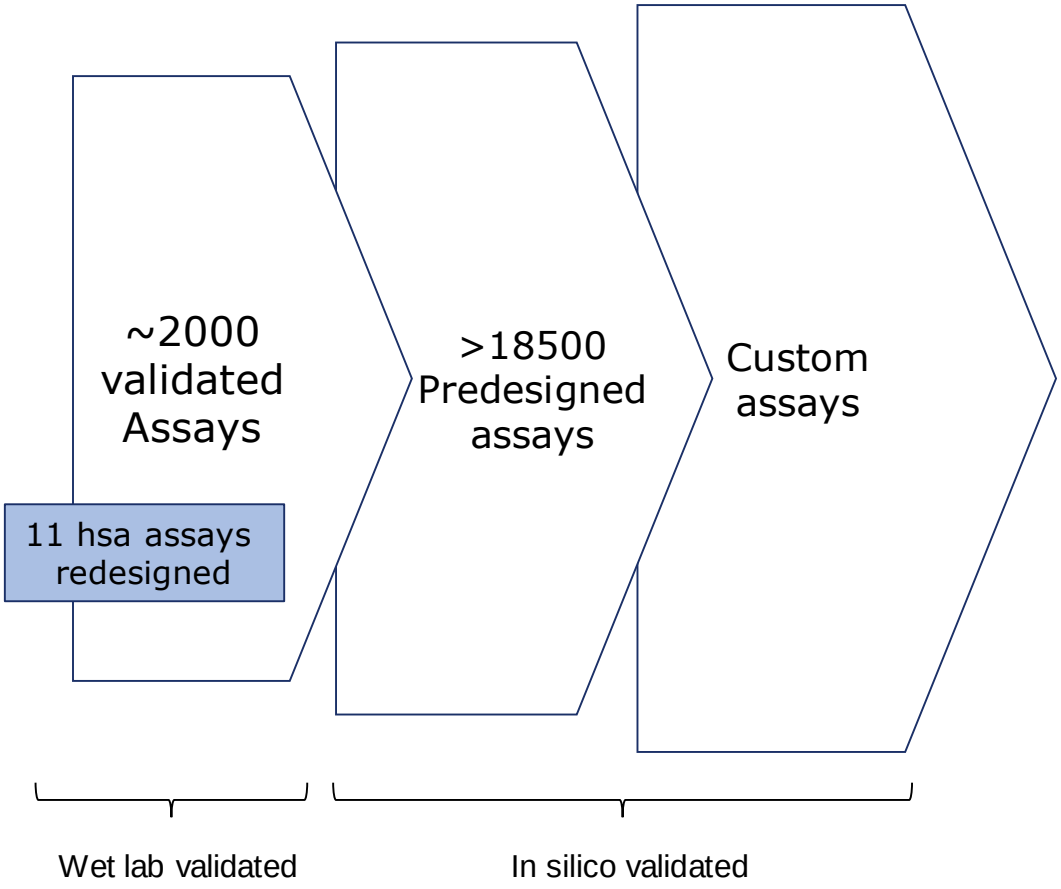


### miRCURY LNA SYBR Green PCR Kit: Improved mismatch discrimination

- Contains an inert blue dye, minimizing errors during reaction setup
- Panels contain a red dye pipetted with the primer pair
- The dyes do not interfere with real-time PCR
- Visual QC

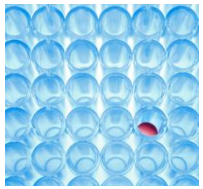
Ensures accurate reaction setup avoiding errors or stress

## New miRCURY LNA miRNA PCR System



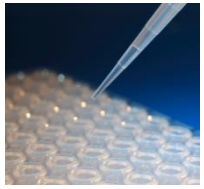

### Vials – PCR Assay

- 200 rxns per vial



### Custom Bulk Plate

- 200 rxns per well

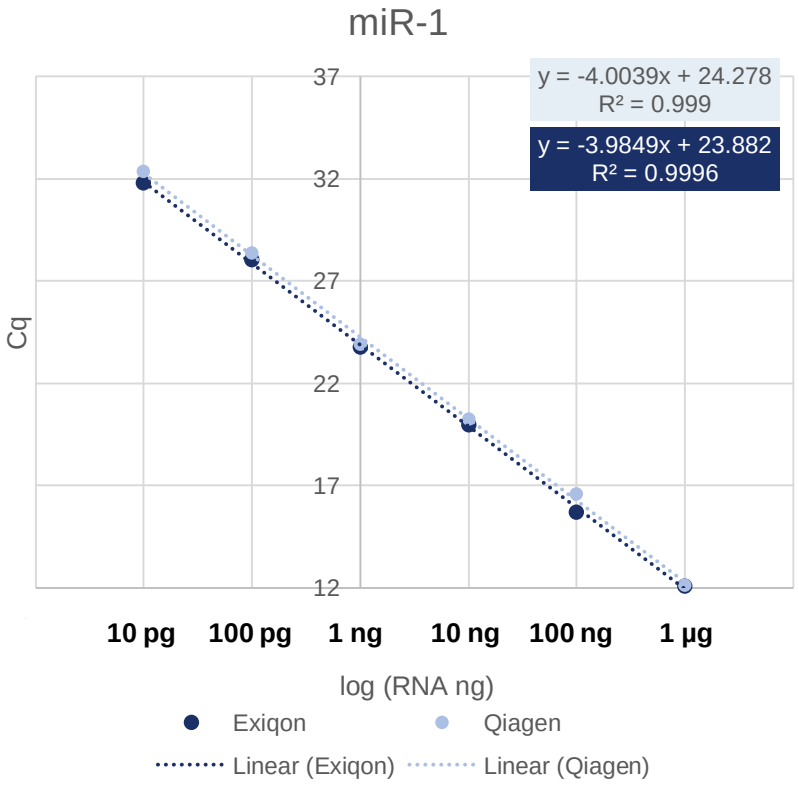


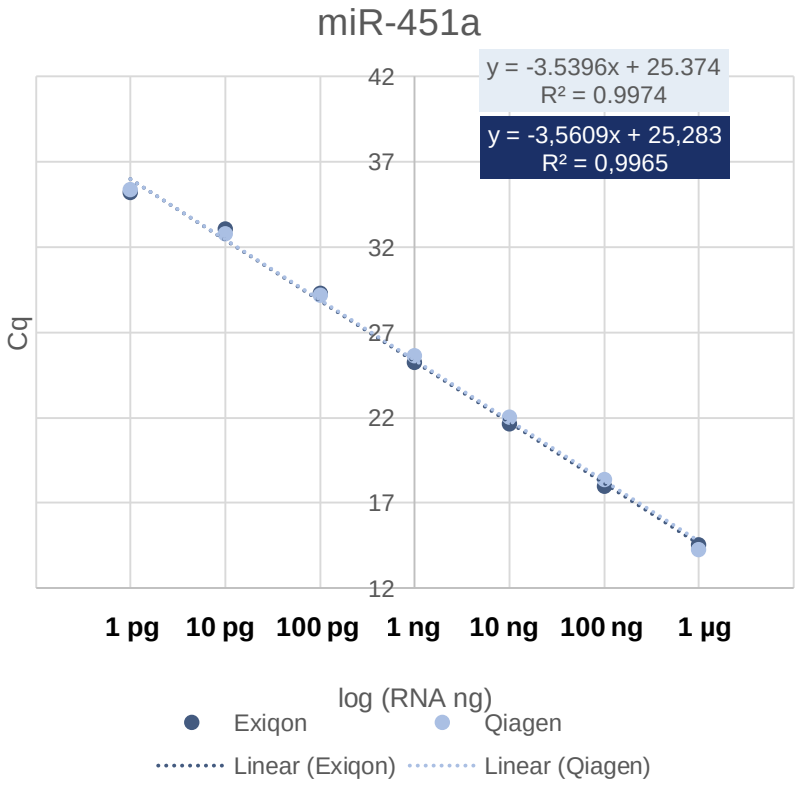
### Custom, miRNome, Focus and QC Panels

- 1 rxn per well

# Superior sensitivity in microRNA detection preserved

## Some examples of dilution curves for single microRNA targets

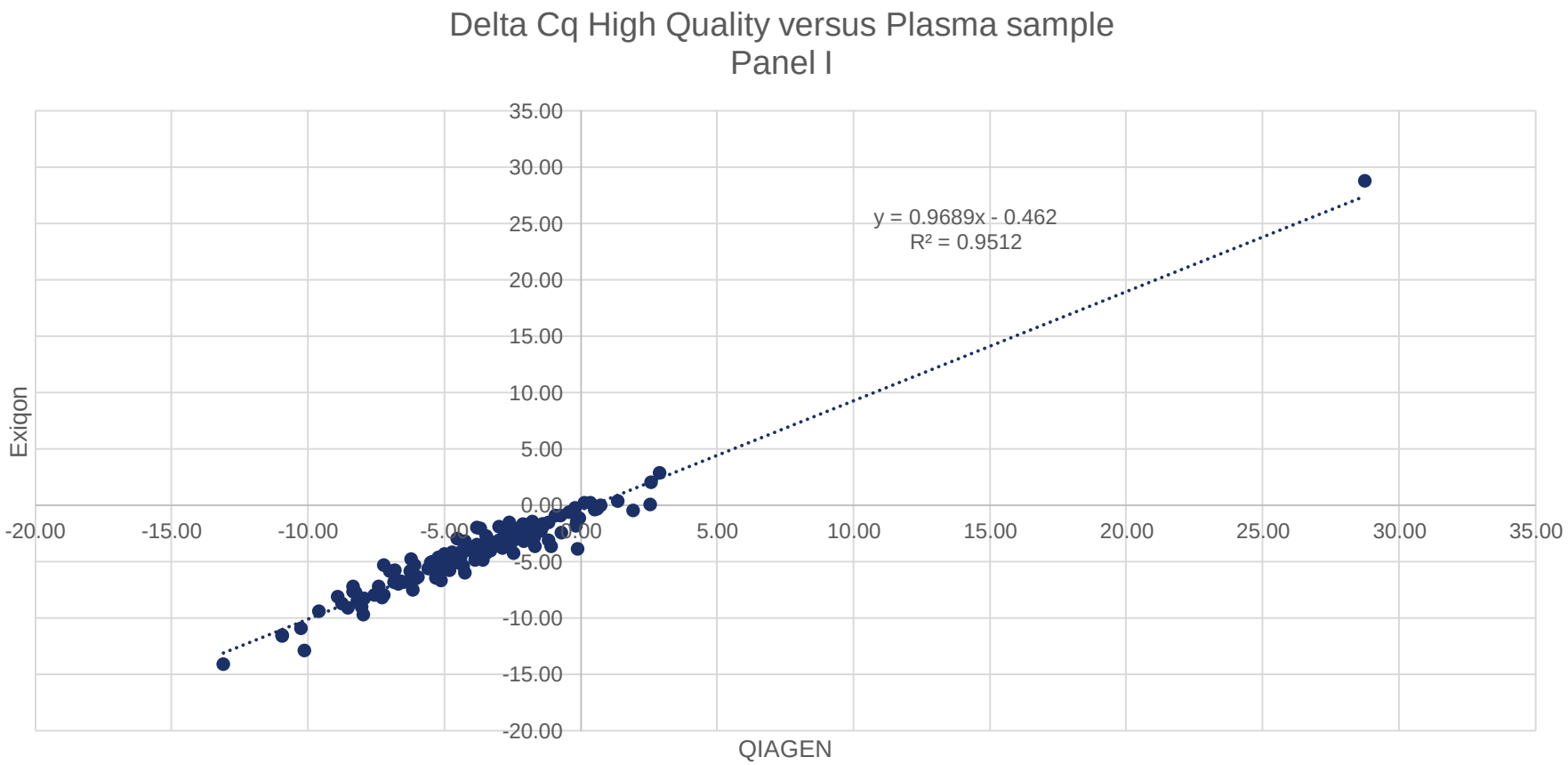



- Similar linear detection of key microRNAs

# Comparison in fold change for individual microRNA

## Comparing two different samples




- Excellent correlation between systems

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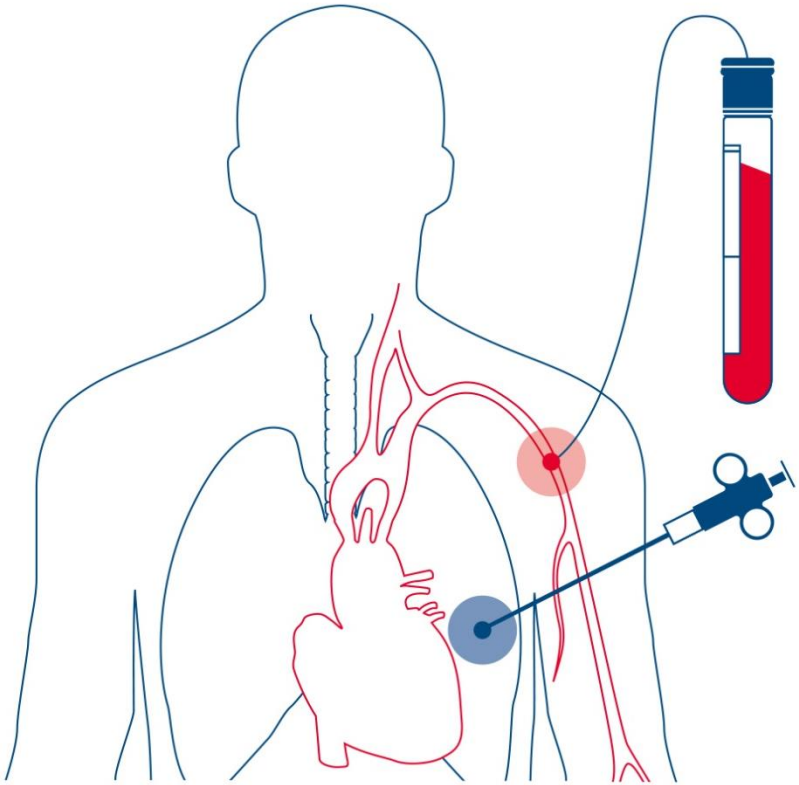
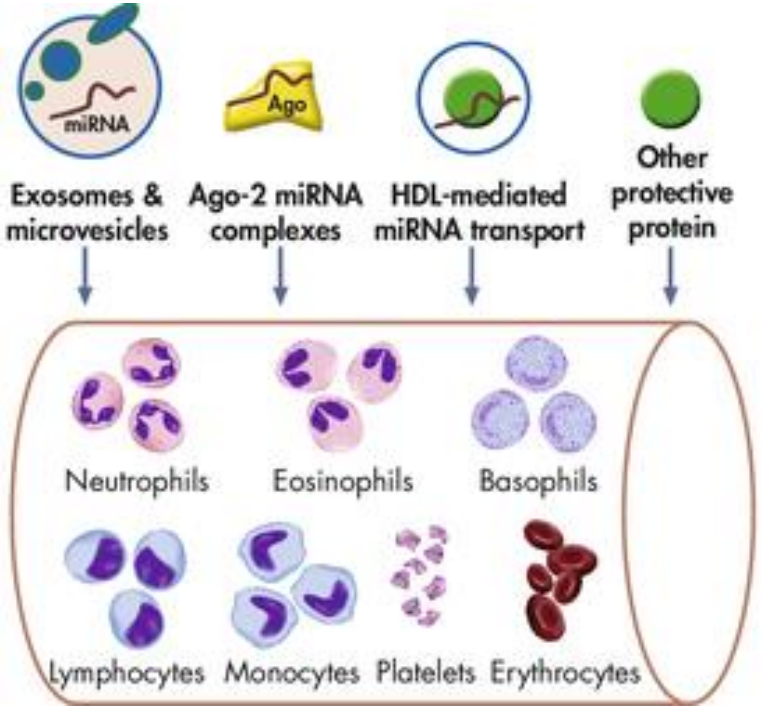
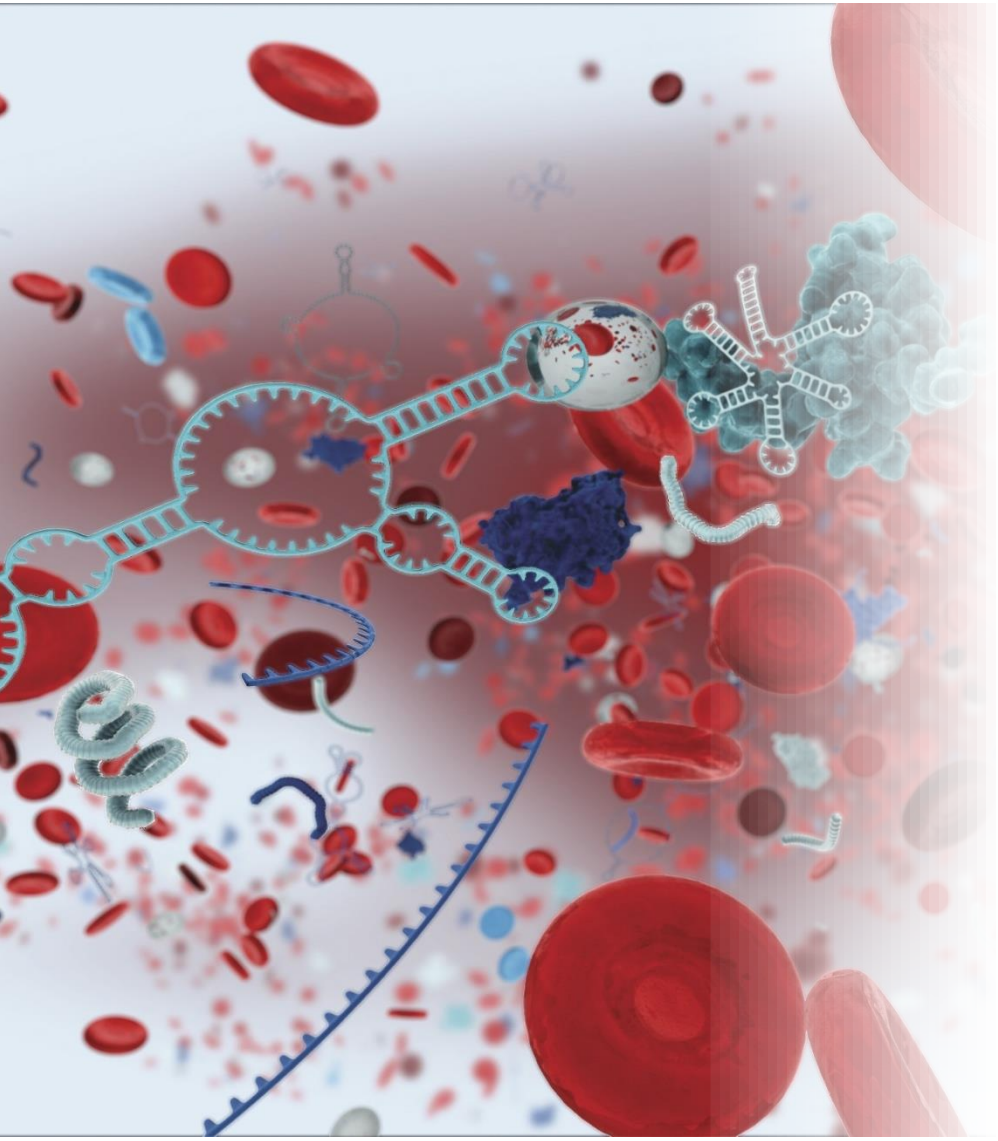
## miRNA can be used as blood-based biomarkers for cancer and other diseases






Source: Arroyo, J.D. et al. (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc. Natl. Acad. Sci. USA 108, 5003.

# Liquid biopsy – Use of biomarker detection in plasma



## Advantages

- Simple sample collection
- Easy integration in hospital/medical workflow
- Many small RNA isolation methods available

(discussed in previous webinar)

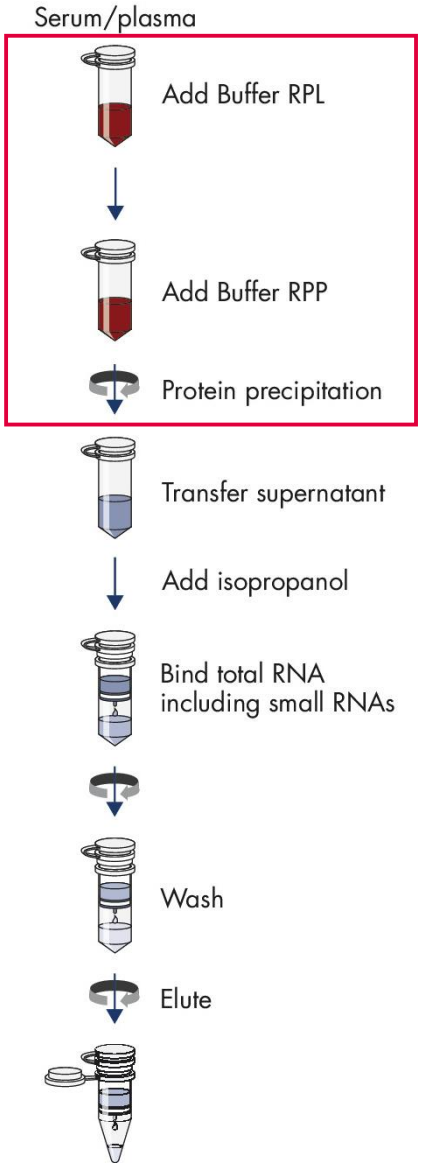
## Challenges

- Tiny amount of RNA
- Inhibition
- Risk of contamination through cellular components or red blood cells – data biased
- Data normalization

# Phenol-free protocol

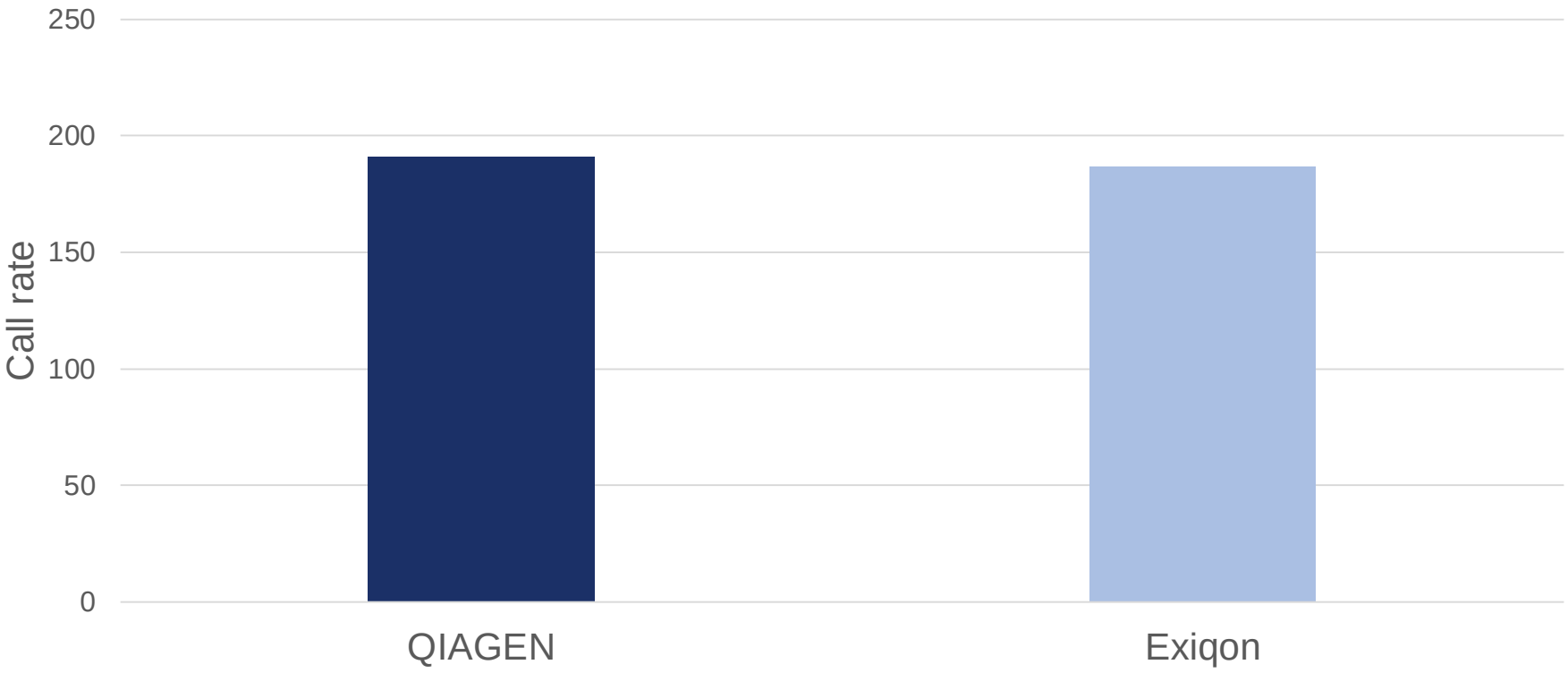
- Instead of phenol
  - Buffer RPL: lysis of proteins and exosomes and inactivation of RNases
  - Buffer RPP: precipitation of proteins and other contaminants
- No phase separation and working under the hood
- MinElute columns to allow for small elution volumes
- Optimal miRNA yields from minimal plasma amounts (200 µl)
- UCP columns for ultraclean eluates
- Automatable on the QIAcube


The QIAcube:
No change from manual spin column procedure



# miRNA biomarkers detection in plasma

## 200 µl plasma analyzed for whole content using screening panels miRNome




miRNeasy Serum/Plasma Advanced Kit
miRCURY LNA miRNA
miRNome PCR Panels v5 I+II

miRCURY™ RNA Isolation Kit – Biofluids
miRCURY™ LNA™ Universal RT microRNA PCR system
miRNome PCR Panels v4 I+II

Very comparable results using the complete QIAGEN miRNA workflow

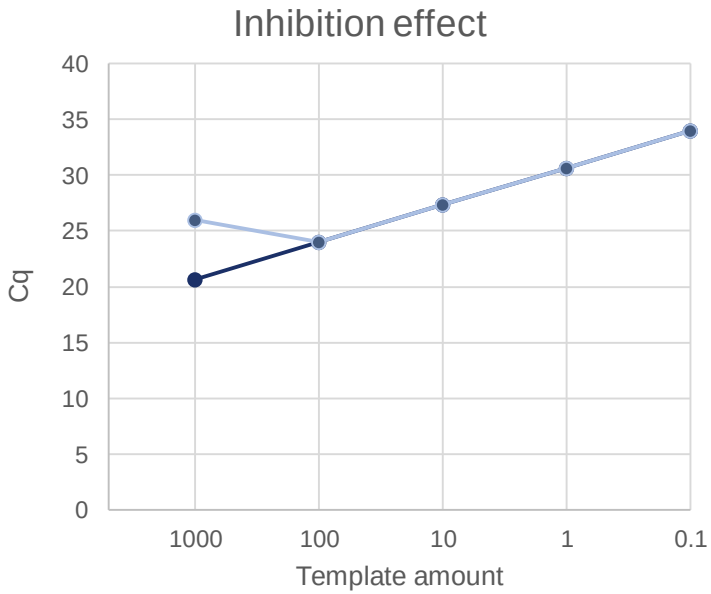
# Controls

## 1 Isolation control

- Check isolation efficiency
- Spike-in templates (in different concentrations)
- Can be detected in downstream application

## 2 RT-control

- Check reverse transcription and qPCR for inhibition
- Spike-in templates (in different concentrations)
- Can be detected in downstream application




## 3 PCR control

- Check PCR amplification for inhibition
- Inter-plate calibrator (if running comparison studies with samples on different plates)

# Available controls

## Artificial controls that can be used as spike-ins

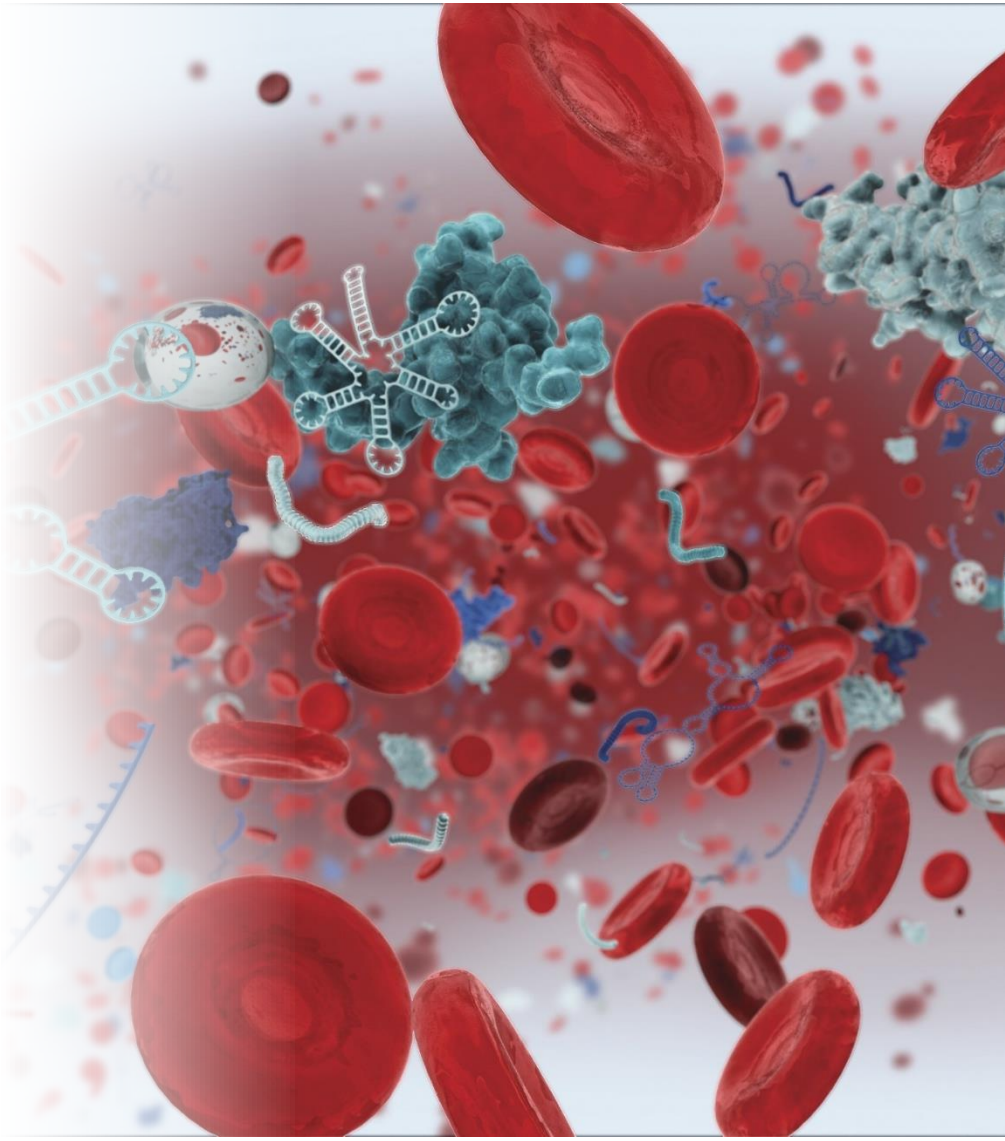
### Controls

| UniSp2, -4 and -5 | UniSp6, cel-miR-39-3p | UniSP3 |
|---|---|---|
| 1 Synthetic RNA targets | 1 Synthetic RNA target | 1 Synthetic DNA target |
| 2 Templates in 3 concentrations (2 logs difference) | 2 Different concentrations | 2 Inter-plate calibrator |
| 3 Add to lysis buffer | 3 Add to RT reaction | 3 Primers & Template are already available on the plate |
| 4 **Check RNA isolation efficiency** | 4 **Check RT and PCR efficiency** | 4 **Amplification only depends on PCR** |

IMPORTANT! These controls cannot be used for normalization purposes!!

# Risk of contamination through red blood cells

The presence of cellular RNA species may disturb the “cell-free” biofluid microRNA profiling experiment resulting in a distorted and non-reproducible profile



## Unique Hemolysis indicator

hsa-miR-451a: highly expressed in RBCs

hsa-miR-23a-3p: unaffected by hemolysis

Check $\Delta C_q$ (miR-23a-3p – miR-451a)

Source: Blondal et. Al. 2013 *Methods* Assessing sample and miRNA profile quality in serum and plasma or other biofluids.

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## GeneGlobe Data Analysis Center

The miRCURY system automated data analysis will be available online soon

- Data analysis
- Normalization

## Service

The QIAGEN Life Science Service Core can help you with your miRNA research

https://www.qiagen.com/us/products/service-solutions/life-science-service-core/

# Thank you for your attention!

## Questions?

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