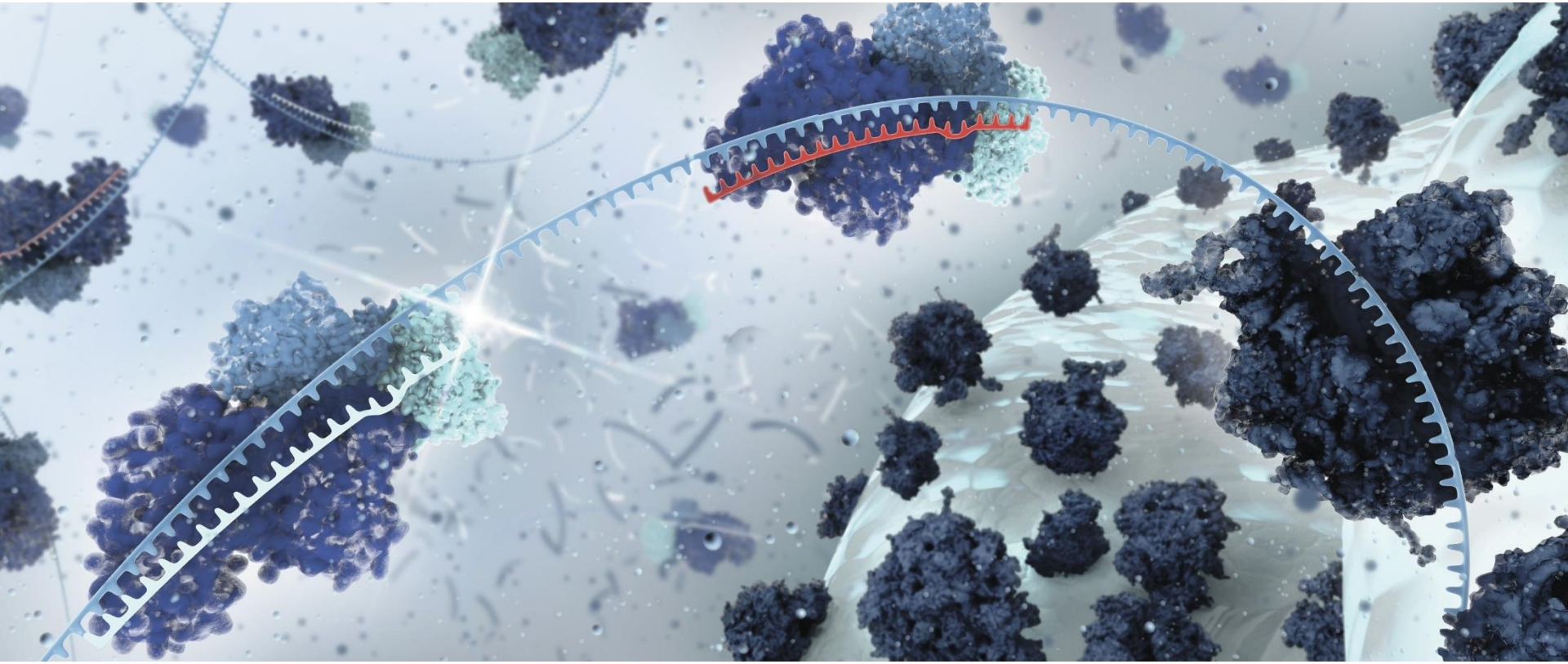
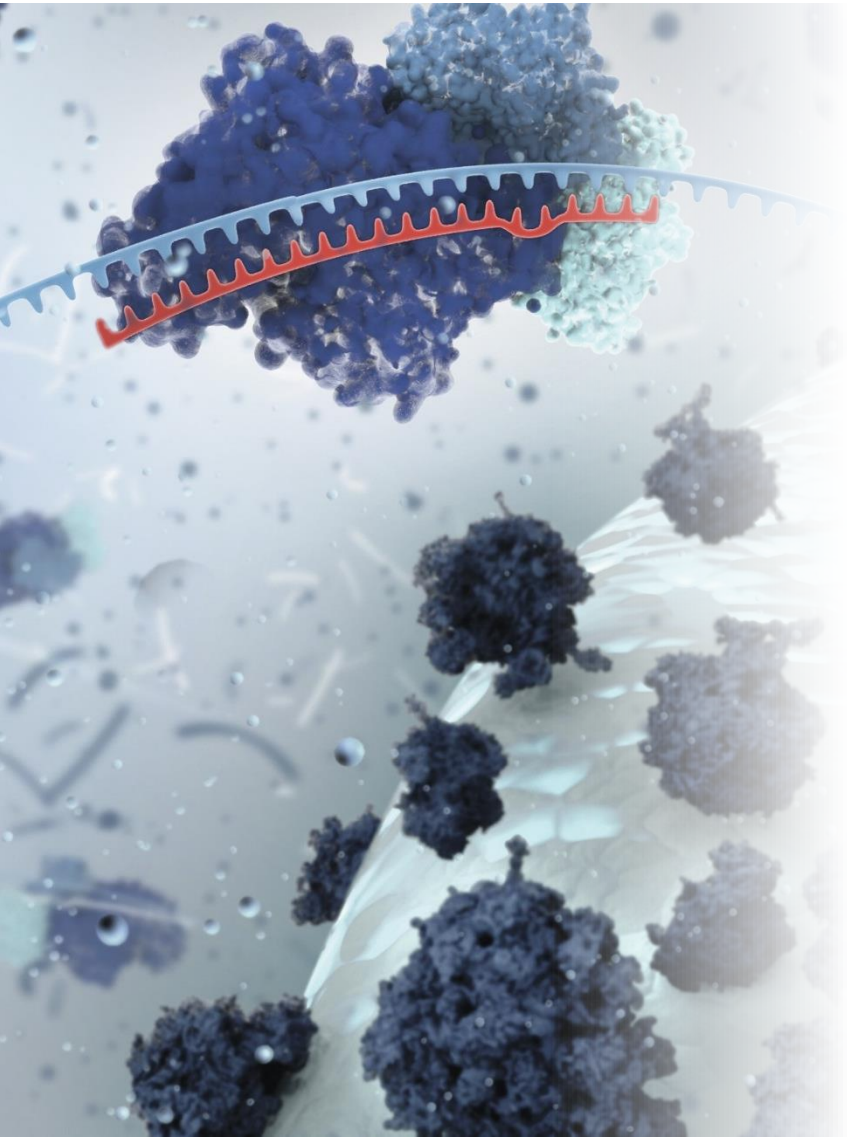


Advancing miRNA Research – Best Practices For Your Experiments

Dr. Verena Schramm, Global Product Manager

Two-part webinar series on circulating RNA biomarkers



Part 1: Advancing miRNA Research – Best Practices For Your Experiment

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

Part 2: Successfully Detect miRNAs Using qPCR With LNA Technology

- Advantages of miRNA detection using qPCR with LNA technology (locked nucleic acids)

Legal disclaimer

QIAGEN products shown here are intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Agenda

1. miRNA introduction
2. Sample types and considerations
3. The new miRNeasy Serum/Plasma Kit
4. Quality control
5. miRNA analysis with NGS
6. Summary and questions

Agenda

1. miRNA introduction
2. Sample types and considerations
3. The new miRNeasy Serum/Plasma Kit
4. Quality control
5. miRNA analysis with NGS
6. Summary and questions

miRNAs: Master regulators of gene expression

miRNAs are ~21 nucleotide, small, non-coding RNAs that are expressed in most tissues

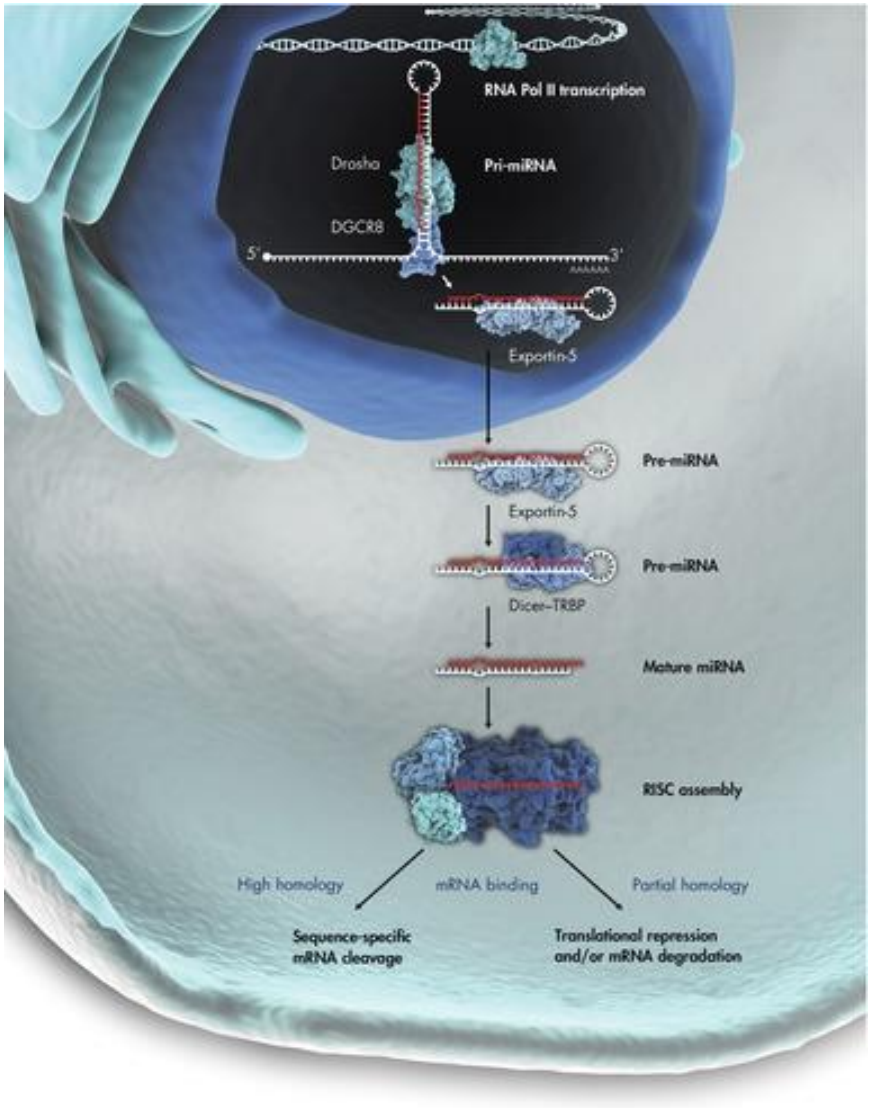
Changes in miRNA expression can be correlated with gene expression changes related to:

- Development
- Differentiation
- Signal transduction
- Infection
- Aging
- Disease

Role in cancer:

miRNAs are associated with cell proliferation, resistance to apoptosis, invasiveness and differentiation in cancer cells

miRNA biogenesis



- Transcribed by RNA Polymerase II, as a long primary transcript (pri-miRNAs), which may contain more than one miRNA
- In the nucleus, pri-miRNAs are processed to hairpin-like, pre-miRNAs by RNAse III-like enzyme Drosha
- Pre-miRNAs are then exported to the cytosol by Exportin 5
- In the cytosol RNAse III-like Dicer processes these precursors to mature miRNAs
- These miRNAs are incorporated in RISC
- miRNAs with high homology to the target mRNA lead to mRNA cleavage
- miRNAs with imperfect base pairing to the target mRNA lead to translational repression and/or mRNA degradation

Why quantify miRNAs?

Differentiate cancer tissue from normal tissue

- Using a signature of altered miRNA expression

Identify the tissue of origin for cancers of unknown primaries

- Using miRNA-based classifier

Early detection of cancers

- Profiling circulating blood or tumor-derived exosomal miRNAs, surpassing the invasive procedures

Distinguishing tumor subtypes

- Using a panel of miRNAs that show differential expression within one cancer type

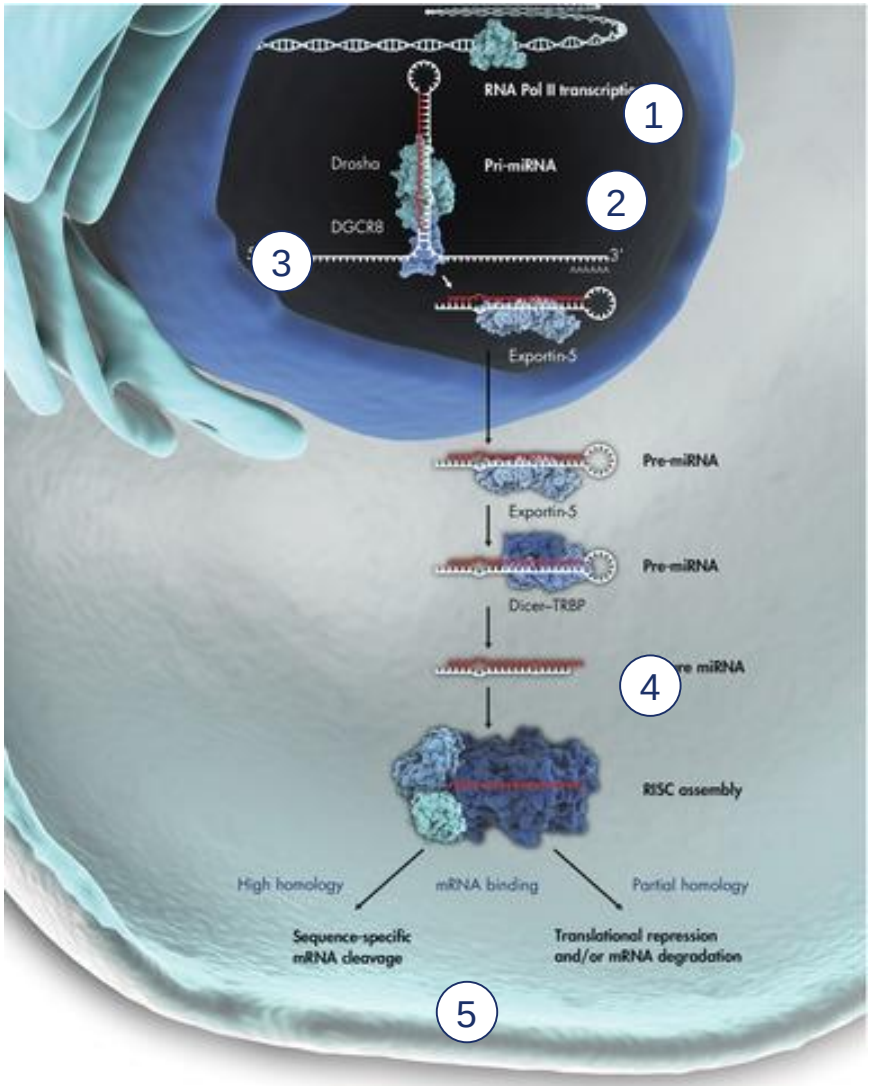
Predict cancer predisposition

- Studying SNPs in the miRNA genes, miRNA binding sites in the target mRNA genes or in the miRNA processing/machinery pathway genes

Source: Paranjape. et. al. (2009) Gut.

Quantifying miRNAs help identify novel expression patterns − both annotated (i.e. in miRBase) and non-annotated (i.e. identified using small RNA sequencing)

Sources of altered miRNA abundance



1. Genomic instability
   - Amplification or deletion
   - Translocation
   - Insertional mutagenesis
2. Altered transcription
   - Methylation
   - Histone modification
   - Transcription factor
3. Drosha processing
4. Dicer processing
5. Loss of miRNA binding site in target mRNA
   - SNP or mutation
   - Alternative splicing
   - Loss or change of 3′-UTR

Disruption of miRNA–mRNA interaction

miRNA expression profiling applications

miRNAs as biomarkers

- Tissue-based miRNA profiling for biomarker discovery
- miRNA profile-based classification of tissues of unknown origin
- Circulating miRNA biomarkers
- Forensics

Understanding gene regulation

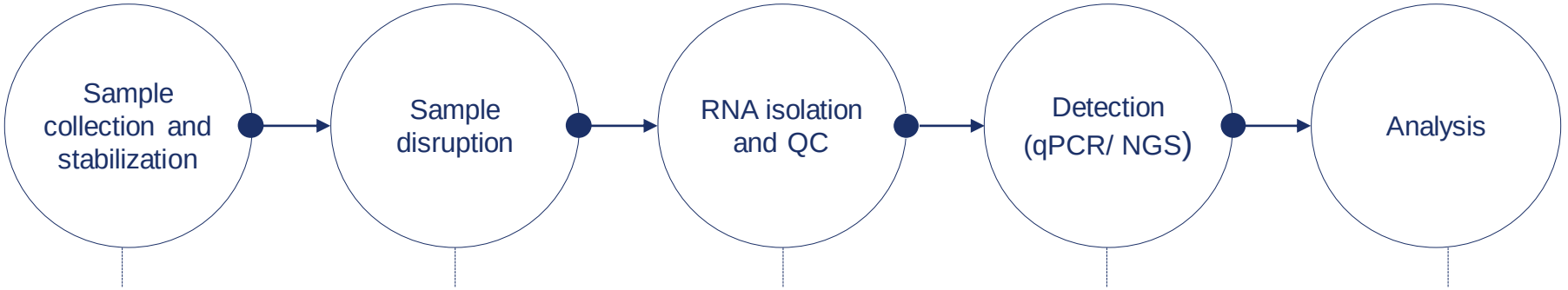
- Developmental biology
- Novel miRNA discovery
- Studying miRNA-mRNA and miRNA-protein interactions
- Total RNA discovery − integrative analyses of miRNAs in the context of gene regulatory networks

Source: Pritchard, C. C. et. al. (2012) Nature Rev. Genet. 13, 358-369

Agenda

1. miRNA introduction
2. Sample types and considerations
3. The new miRNeasy Serum/Plasma Kit
4. Quality control
5. miRNA analysis with NGS
6. Summary and questions

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

Sample preparation: A total RNA solution for every sample type

Cells, fresh tissue and frozen tissue

- miRNeasy Mini Kit
- miRNeasy Micro Kit
- miRNeasy 96 Kit

FFPE tissue

- miRNeasy FFPE Kit

Fluids (serum, plasma, urine, CSF and saliva)

- miRNeasy Serum/Plasma Advanced Kit

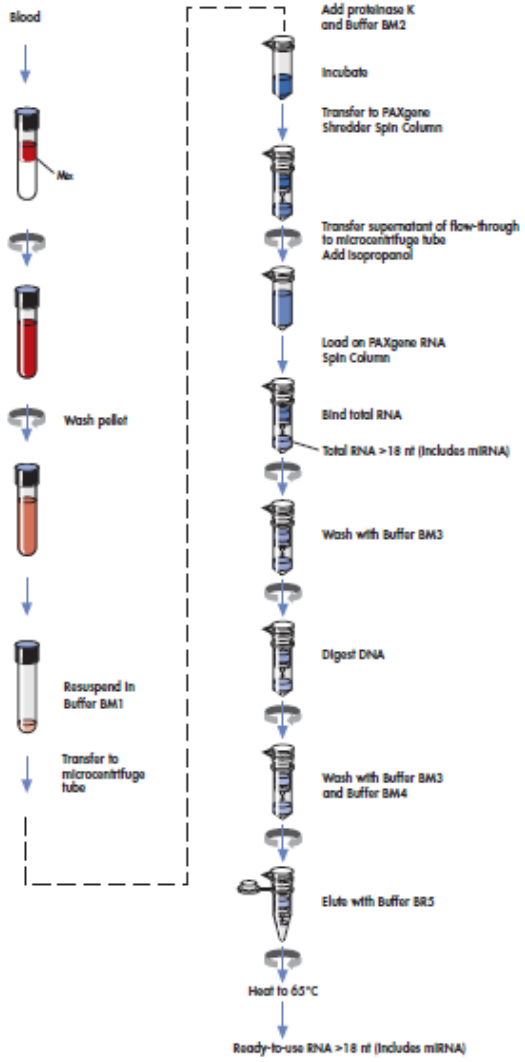
Exosome enrichment/miRNA isolation from serum/plasma

- exoRNeasy Serum/Plasma Midi Kit
- exoRNeasy Serum/Plasma Maxi Kit

PAXgene Blood miRNA Kit

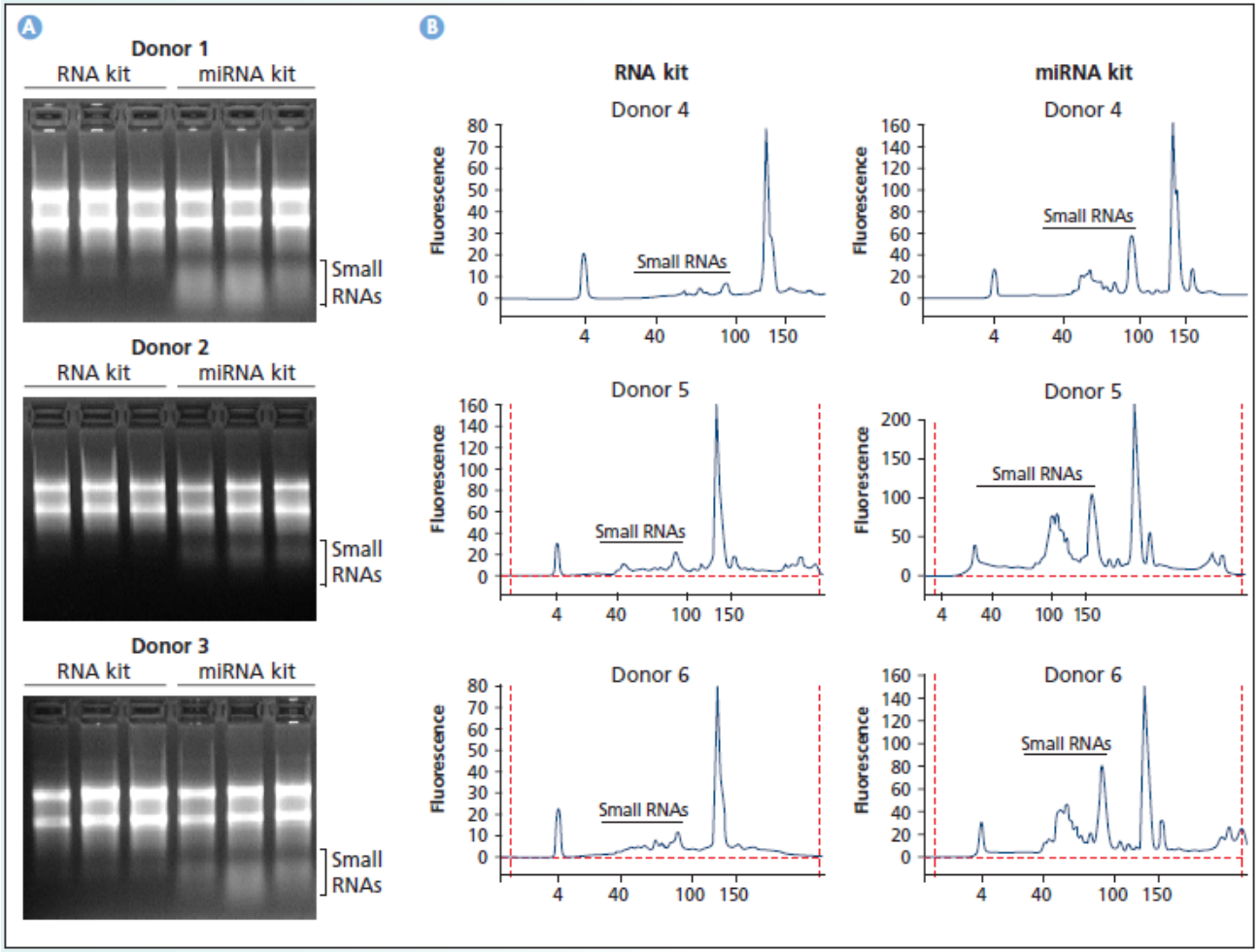
PAXgene Tissue miRNA Kit

Effective purification of intracellular RNA including miRNA, from whole blood



- Collection, stabilization, transport & storage in PAXgene Blood RNA Tubes
- miRNA relevant for liquid biopsy analysis is well protected from degradation in plasma
- Challenges are
  - 2.5 ml blood draw volume
  - RNA stabilization for up to 3 days at 15-25°C, up to 5 days at 2-8°C or at least 11 years at -20 or -70°C (studies ongoing)
- Standardized sample processing prior to analysis
- Purification of total RNA, including RNA >18 nucleotides (including miRNAs)
- Integrated DNase treatment for removal of gDNA
- Purification automatable on the QIAcube

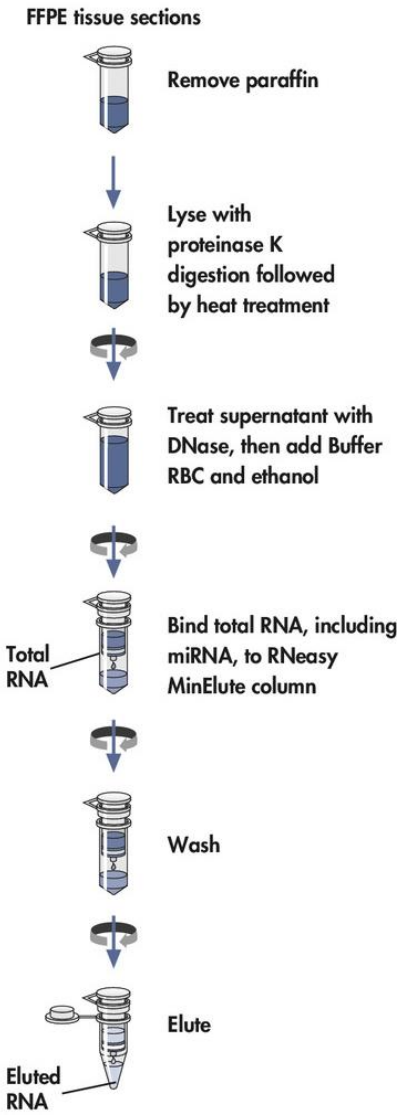
Enrichment of miRNAs



High yields of small RNA species. Blood was collected in PAXgene Blood RNA Tubes and stored for 20–24 hours at room temperature before freezing at –15 to –30°C. RNA was purified using the PAXgene Blood RNA Kit (**RNA kit**) or the PAXgene Blood miRNA Kit (**miRNA kit**). **A** Purified RNA was analyzed by agarose gel electrophoresis. **B** Purified RNA was analyzed using the Agilent® Small RNA LabChip. Small RNA species were significantly enriched when using the PAXgene Blood miRNA Kit.

Dedicated kit to maximize miRNA yields from FFPE tissue

- Proteinase K and heat treatment for tissue digestion
- Centrifugation to remove cell debris and crosslinked DNA
- Additional DNase treatment to remove remaining degraded DNA
- Buffer RBC and ethanol to optimize binding conditions to the spin column membrane
- Spin column protocol to maximize miRNA yields without further degrading RNA

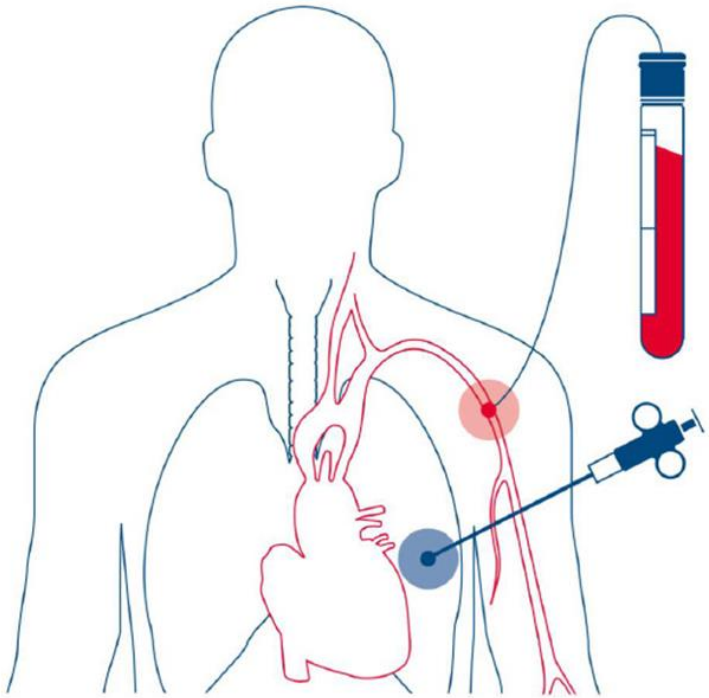


Agenda

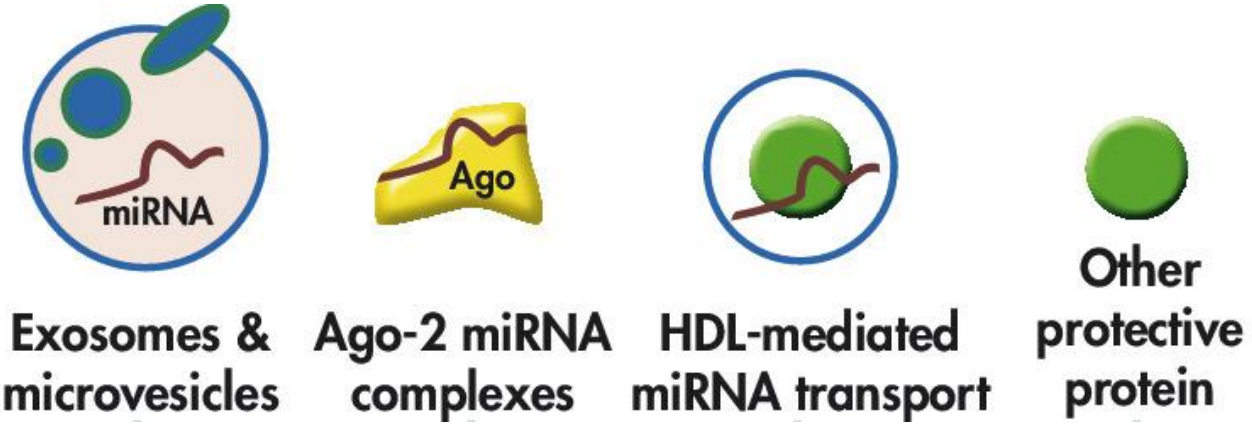
1. miRNA introduction
2. Sample types and considerations
3. New miRNeasy Serum/Plasma Advanced Kit
4. Quality control
5. miRNA analysis with NGS
6. Summary and questions

miRNA isolation from serum/plasma

- Liquid Biopsy - a minimally invasive method that can be used to help detect molecular, disease-related biomarkers in body fluids
- miRNA relevant for liquid biopsy analysis is well protected from degradation in plasma
- Challenges are
  - low RNA content
  - high inhibitor amount in serum/plasma affecting downstream assays



Cell-free miRNA in plasma or serum



miRNA in serum/plasma is quite well protected from degradation

- Bound to protein complexes mainly Ago-2 and lipoproteins
- Contained within exosomes and microvesicles

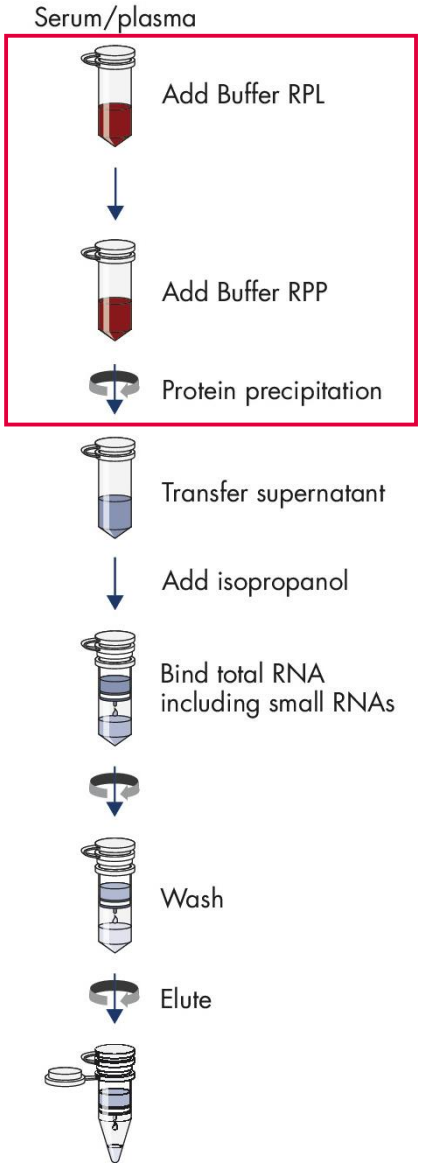
miRNA isolation method from serum/plasma needs to be highly efficient in lysing proteins and lipid bilayer membranes

1) Footnote / 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.

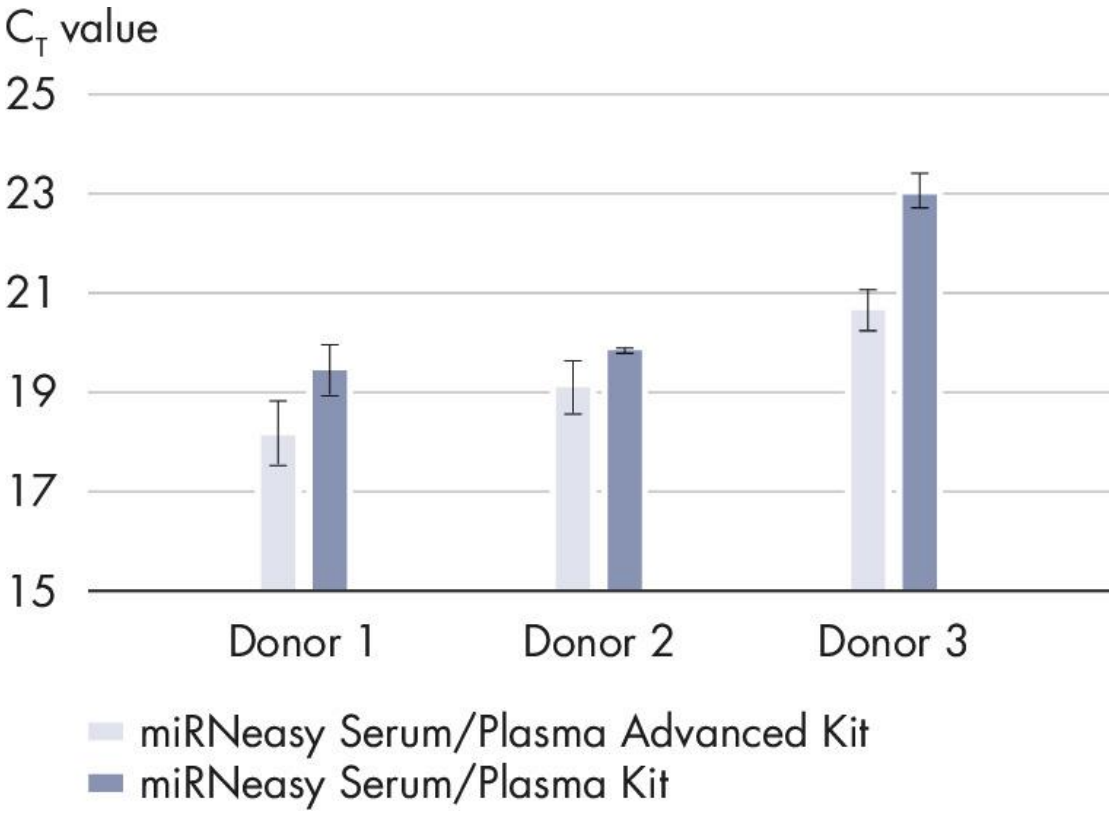
Phenol-free protocol

- 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



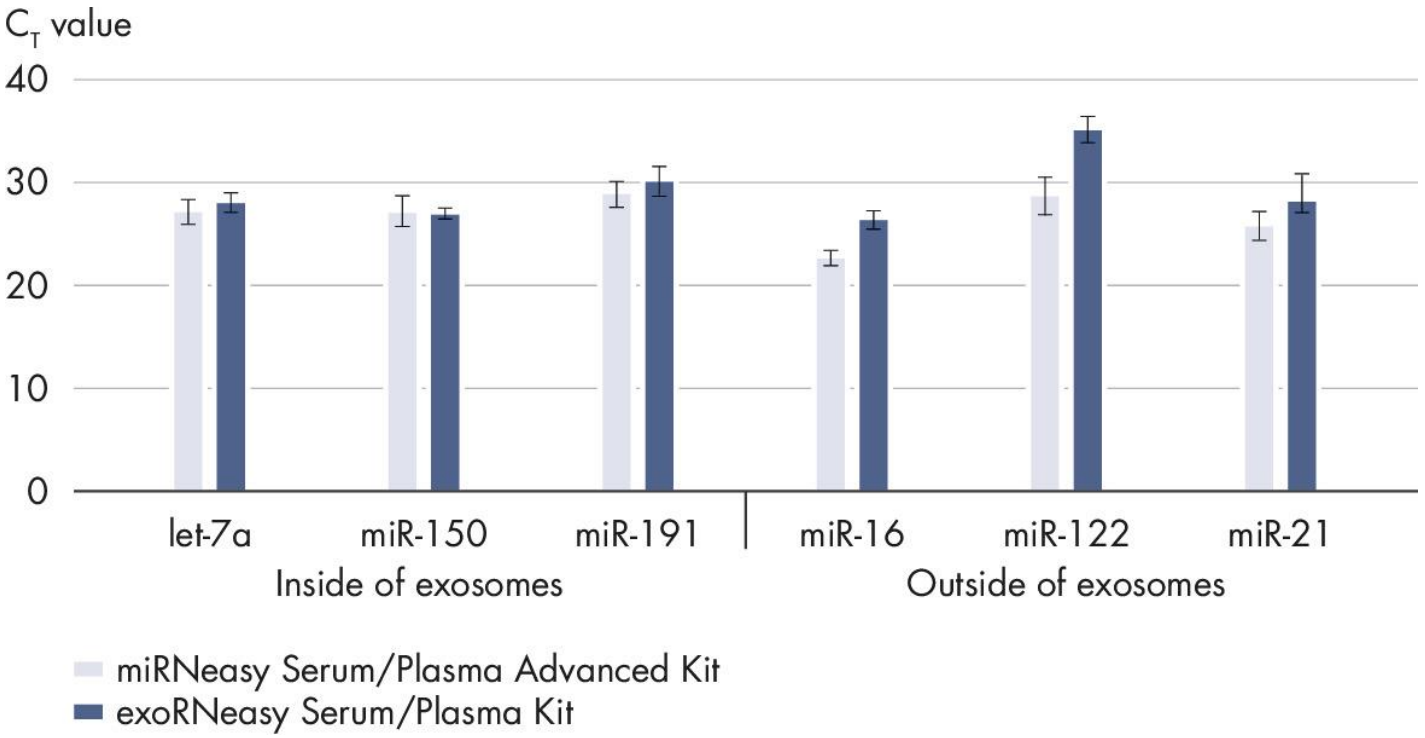
No phenol and no compromise on miRNA yields



- Three different samples
- miR16 as target in miScript qPCR Assay

Compared to the miRNeasy Serum/Plasma Kit, the new Advanced Kit provides better or comparable yields

Analysis of miRNA content inside and outside of plasma EVs

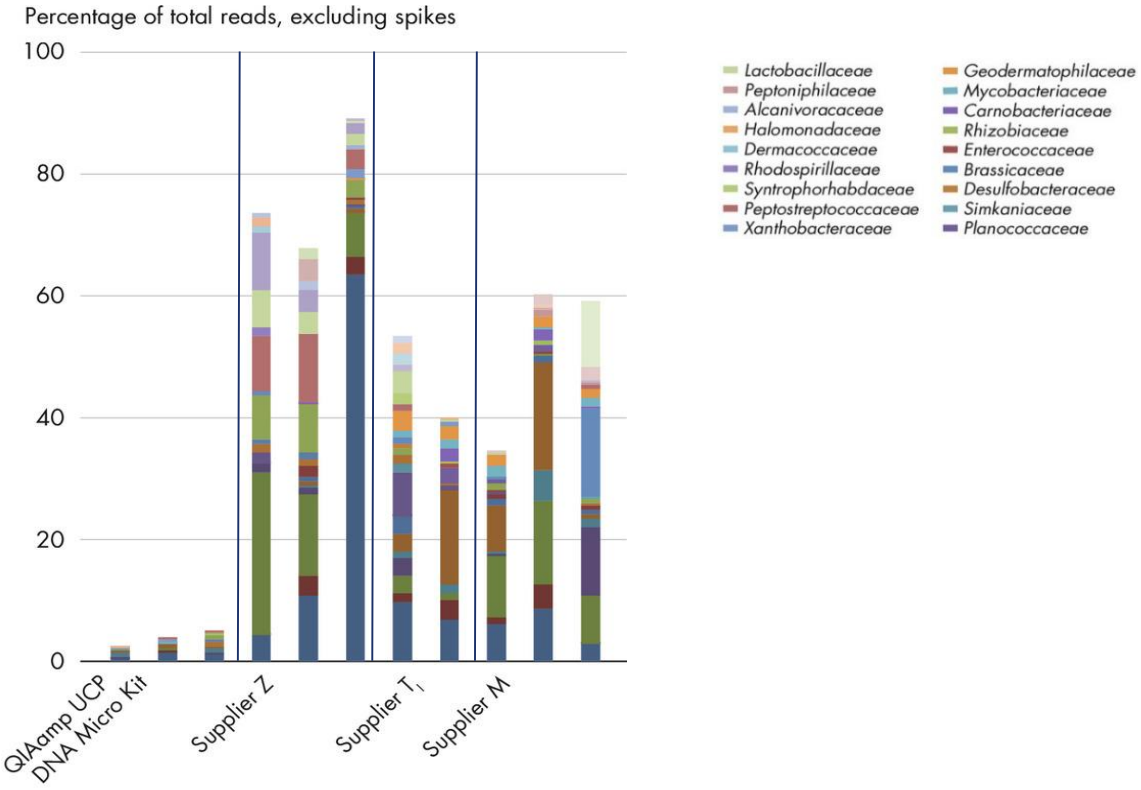


- Twenty different samples; miScript Primer Assay
- This data have been generated within the CANCER ID project, funded by the Innovative Medicines Initiative (2) Joint Undertaking (JU) under grant agreement No. 115749
- The JU receives support from the European Union's Horizon 2020 research and innovation program & EFPIA

Efficient lysis of extracellular vesicles to release RNA content

UCP column concept

- NGS provides an unbiased way to look at a sample's nucleic acid content
- Clean eluates containing only the desired nucleic acids are essential to maximize NGS outcome and usable data
- QIAGEN developed a special treatment for spin columns to ensure minimal residual nucleic acid load – the UCP column concept

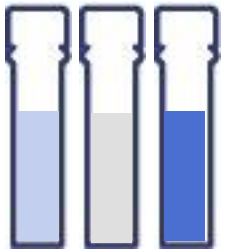


Agenda

1. miRNA introduction
2. Sample types and considerations
3. The new miRNeasy Serum/Plasma Kit
4. Quality control
5. miRNA analysis with NGS
6. Summary and questions

Main RNA quality parameters also apply for liquid biopsy

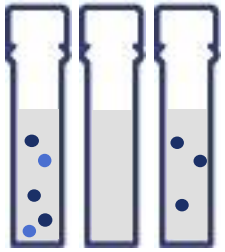
Quantity



How much RNA is in the tube? Will I have enough material?

- Over- or underestimation of RNA amounts can lead to wrong ratios of sample and enzymes and inhibitory effects during NGS library preparation resulting in suboptimal yield of NGS library molecules

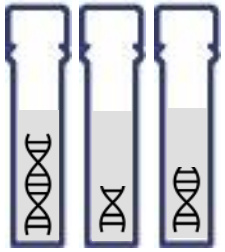
Purity



What is really in my tube? Could impurities interfere with my assay?

- Phenol, ethanol, salts, etc. can jeopardize sensitivity and efficiency of downstream enzymatic reactions
- Large amounts of unwanted ssDNA or dsDNA template lead to overestimation of the target and interferes with downstream applications

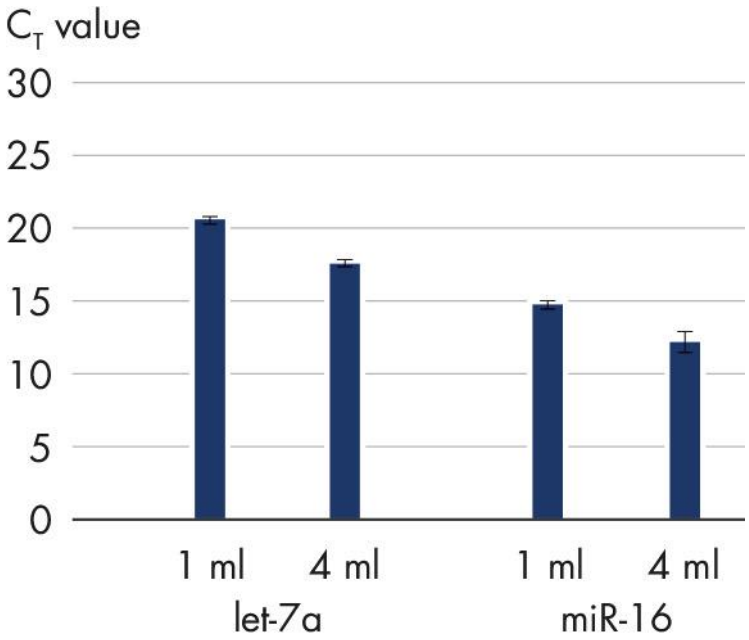
Integrity



Does my RNA sample have the correct size distribution? Has RNA been degraded?

- Poor integrity RNA samples can affect PCR or sequencing reactions and lead to errors in replication and/or lower yields e.g., irrelevant C_T values
- Degraded samples can produce false negative results

Assessing RNA quantity



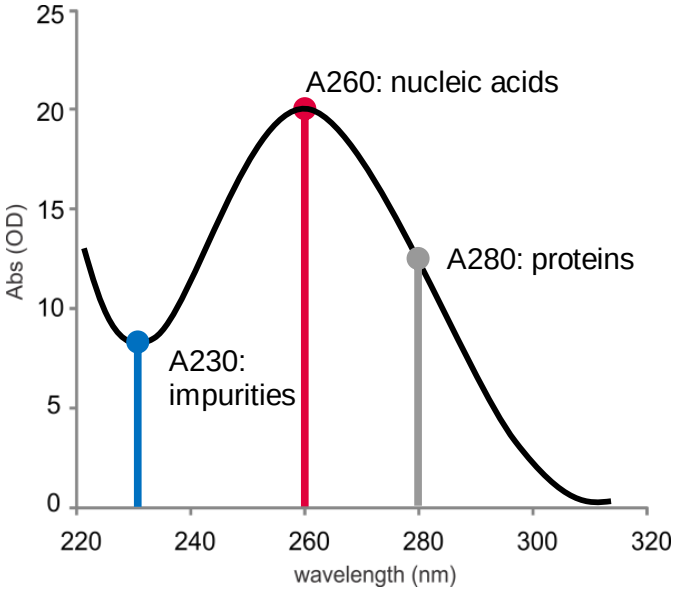
An example qRT-PCR for miRNA quantification

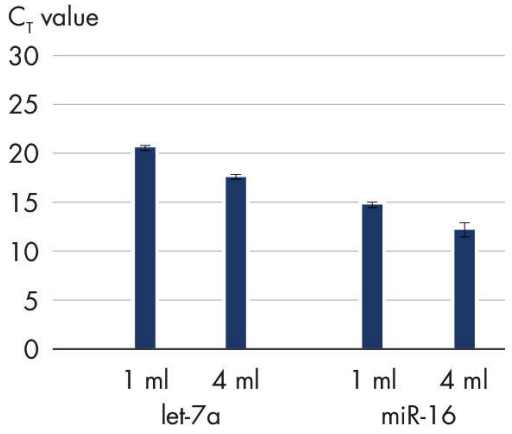
- Usual quantity measurement: A_{260}
- An absorbance of 1 unit at 260 nm corresponds to 44 µg/ml of RNA (at neutral pH)
- Not reliable with low concentrations (~20 ng/µl) or small RNAs < 200 bp
  - Cell-free RNA concentrations in serum, plasma or other body fluids are very low
- For short RNAs like miRNAs, fluorometric quantification (e.g., using Qubit or Nanodrop) is often unreliable
- We recommend to use quantitative RT-PCR instead

Determination of RNA quality – purity

A_{260}/A_{280} provides an estimate of RNA purity

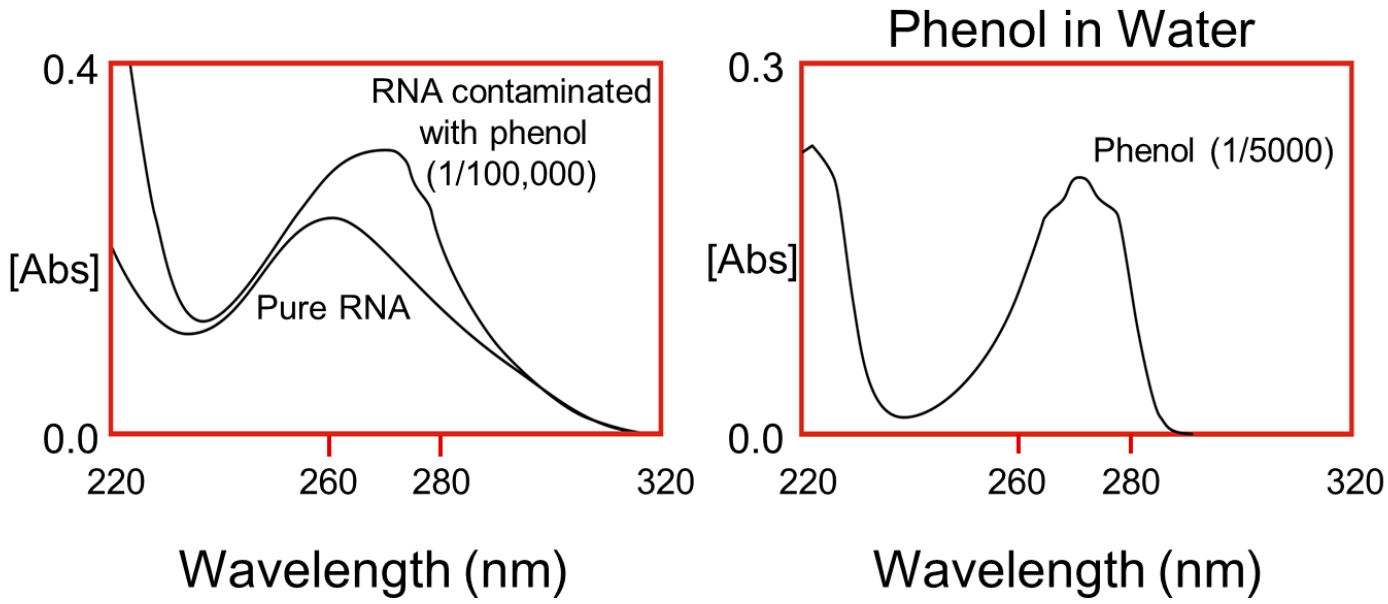
- A_{260}/A_{230} estimates impurities like salts in your sample
- A_{260}/A_{280} should be >1.8 to avoid downstream assay inhibition
- Always calibrate the spectrophotometer with the same solution used for elution
- For small RNAs, qRT-PCR can be used to measure if an increase of miRNA is observed with an increase in sample volume
- DNA contamination:
  - QIAzol and RNeasy technology remove the majority of DNA
  - Design primers to be RNA specific or use kits that remove DNA like QuantiNova Reverse Transcription Set
  - Optional on-column DNase treatment





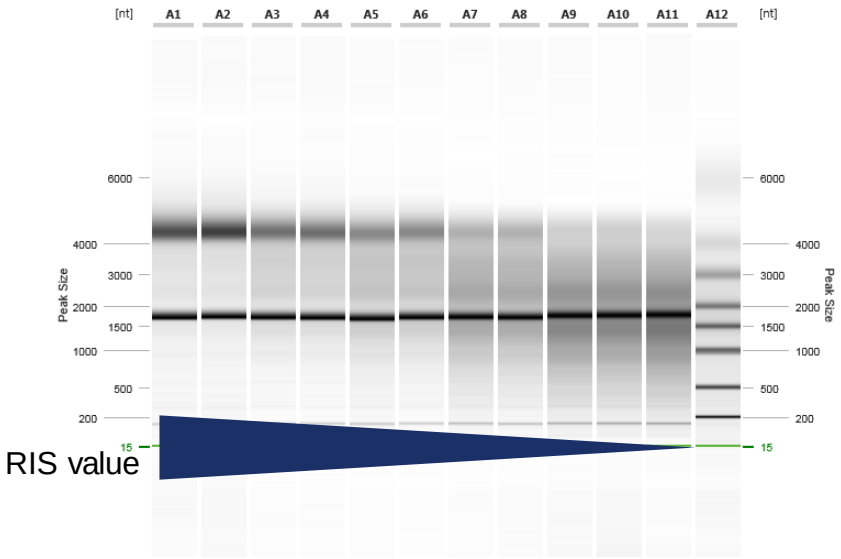
Effect of phenol on UV absorbance

- Phenol-chloroform treatment is effective at removing contaminants like DNA and proteins
- Careful pipetting of the aqueous phase and avoiding phenol carry-over is important for accurate RNA QC
- Phenolic solutions absorb in the UV range at 230 nm with a maximum at ~270 nm



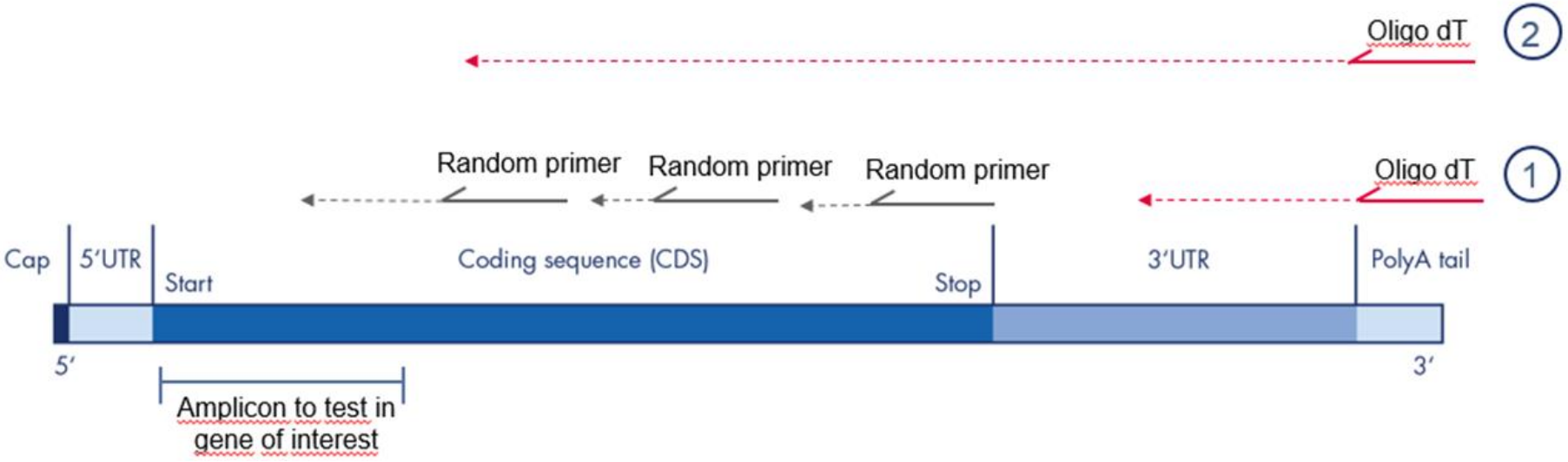
Phenol contamination imitates higher RNA content of the sample

RNA integrity testing based on mRNA



- Usual assessment: RIN value; appearance of rRNA bands (Bioanalyzer or QIAxcel systems)
  - Range: 1–10
  - Suitable for downstream assays like NGS: RIN 7–10
  - RIN is dependent on sample type
- Cell-free RNA is limited in amount and mainly consists of small RNAs <100 bp
  - rRNA bands on agarose gel or in Bioanalyzer / QIAxcel is usually cellular RNA contamination
  - RNA integrity of liquid biopsy samples cannot be assessed via agarose gel or Bioanalyzer / QIAxcel
- For limited amounts of RNA like cell-free RNA, we usually determine the ΔC_T between a random priming mix and oligo dT priming mix

Testing RNA integrity for limited samples – oligo-dT/random priming



- Based on poly-A mRNA
- cDNA synthesis with two different strategies:
  - Random primer/oligo-dT
  - Oligo-dT
- In second step, amplicon to test is detected through PCR
- Calculation of ΔCt: ΔCt = oligo-dT – random priming/oligo-dT
- The larger the ΔC_T, the less intact the RNA

Agenda

1. miRNA introduction
2. Sample types and considerations
3. The new miRNeasy Serum/Plasma Kit
4. Quality control
5. miRNA analysis with NGS
6. Summary and questions

QIAseq miRNA overview

What is the kit?

miRNA-focused NGS library prep kit and integrated bioinformatics/data analysis solution

- Compatible with Illumina sequencers

What can be done with the sequencing data?

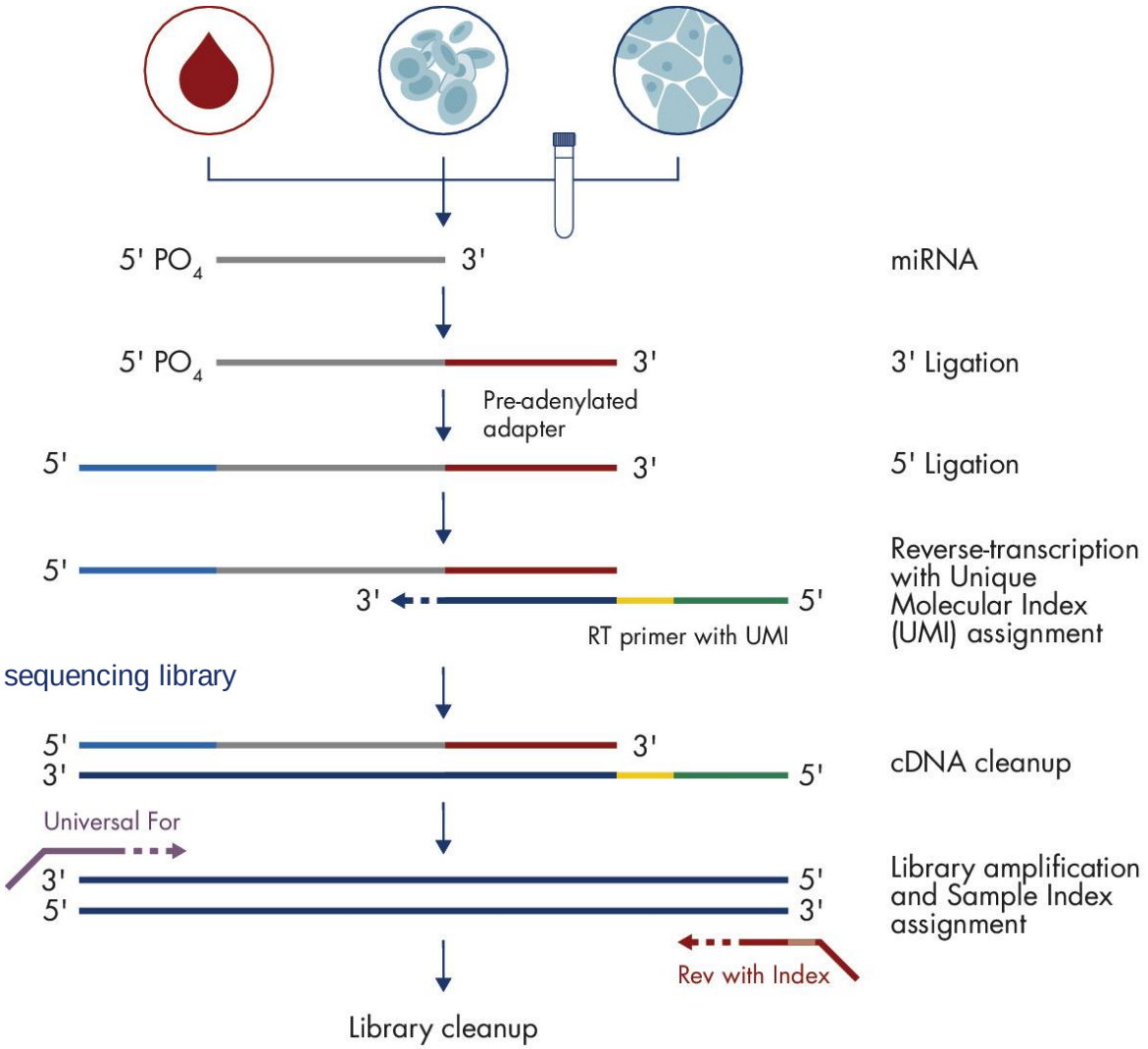
- Differential expression calculations of miRNA from highly multiplexed samples
- Novel miRNA discovery

What are the distinguishing features of the prep kit?

- Gel-free, rapid workflow
- Broad RNA input: 1−500 ng
  - No adapter dimers at any RNA input amount
- Library prep from serum, plasma, biofluids, cells and tissues (any species)
- Integrated Unique Molecular Index (UMI) technology
- Highly optimized chemistry
- All-in-one-box solution

QIAseq miRNA Library Kit: Unparalleled miRNA-focused sequencing for robust miRNA quantification and discovery

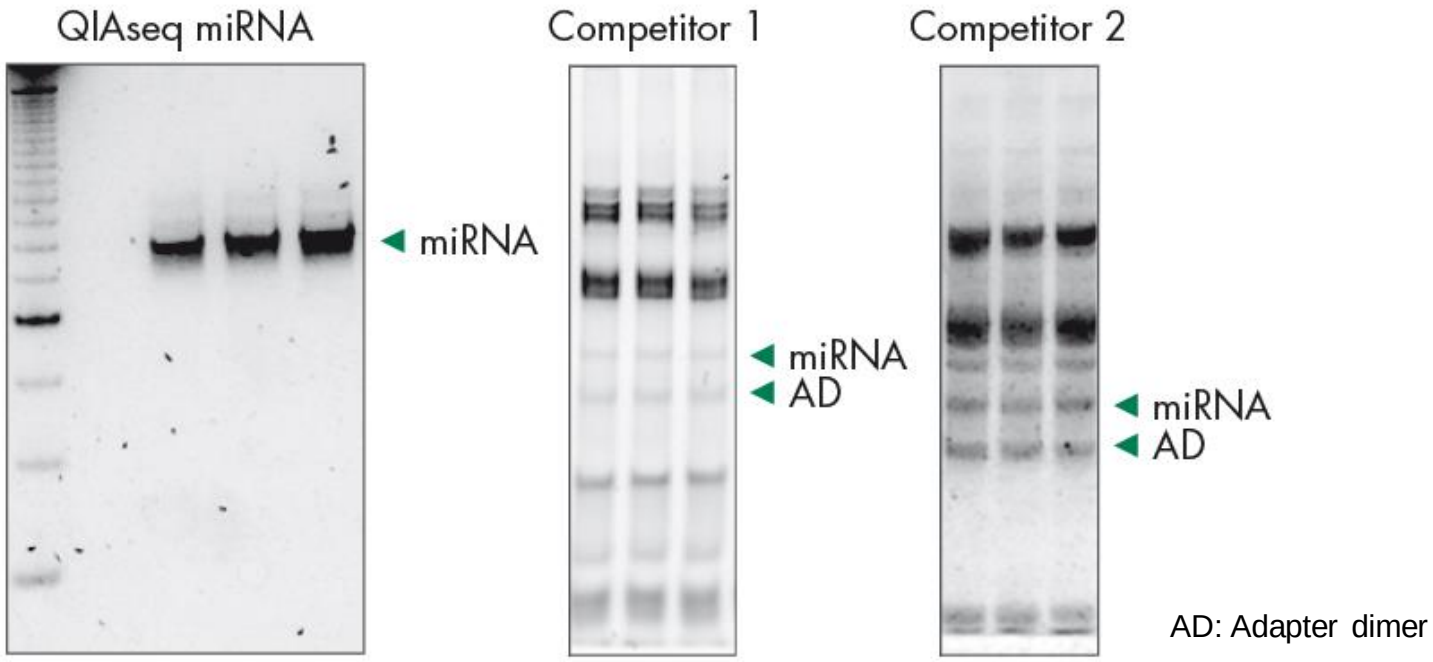
QIAseq miRNA: Library construction



Elimination of adapter dimer from sequencing library

Library pre-seq QC, determining library concentration, preparation for sequencing and data analysis

QIAseq miRNA vs. two competitor (C) kits

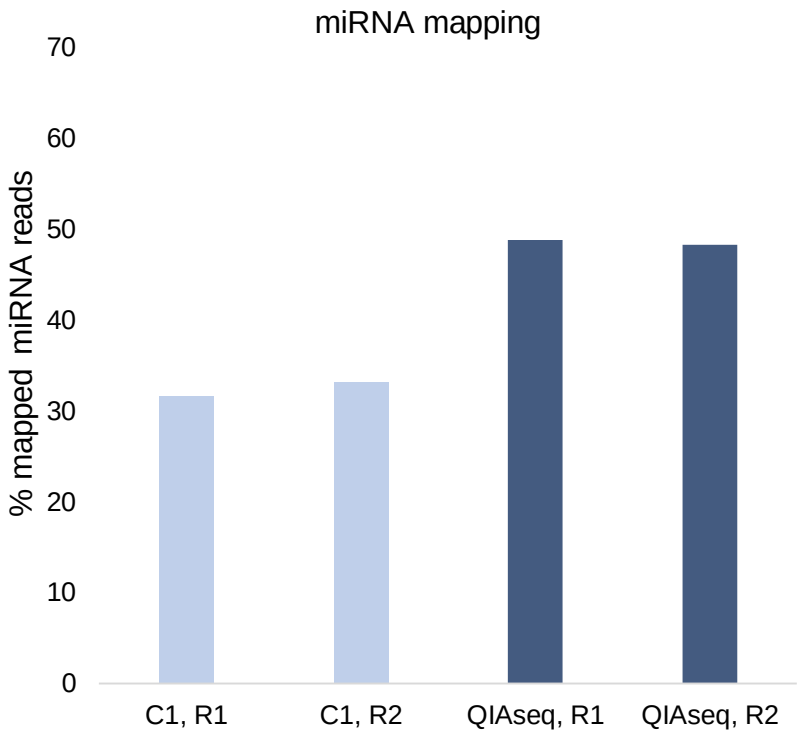


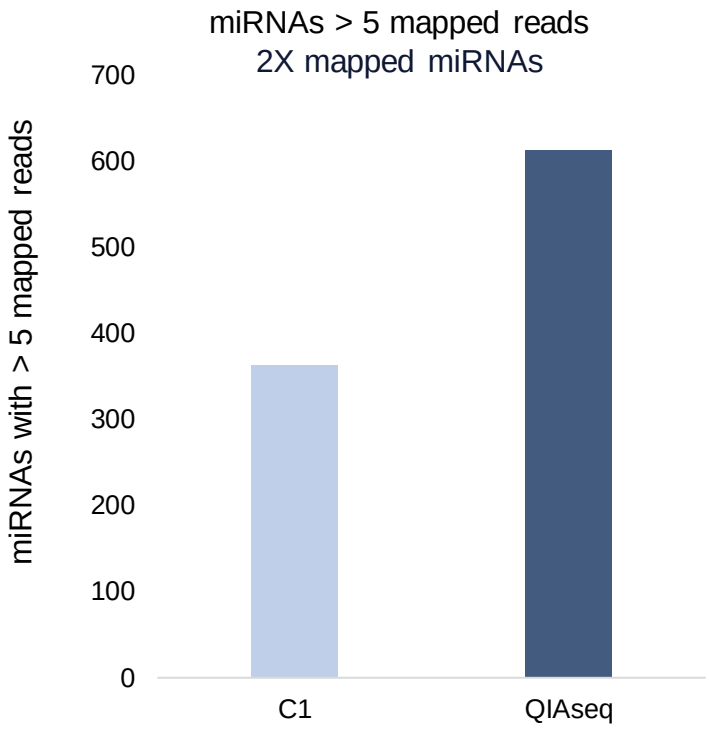
PAGE gel after standard library prep protocol

RNA amounts: 100 ng (QIAseq miRNA), 1 µg (C1) and 100 ng (C2)

- QIAseq miRNA, generates a robust, specific miRNA library with negligible background. Other commercial options are fraught with side-products, including adapter dimers

QIAseq miRNA Library Kit outperforms the competition



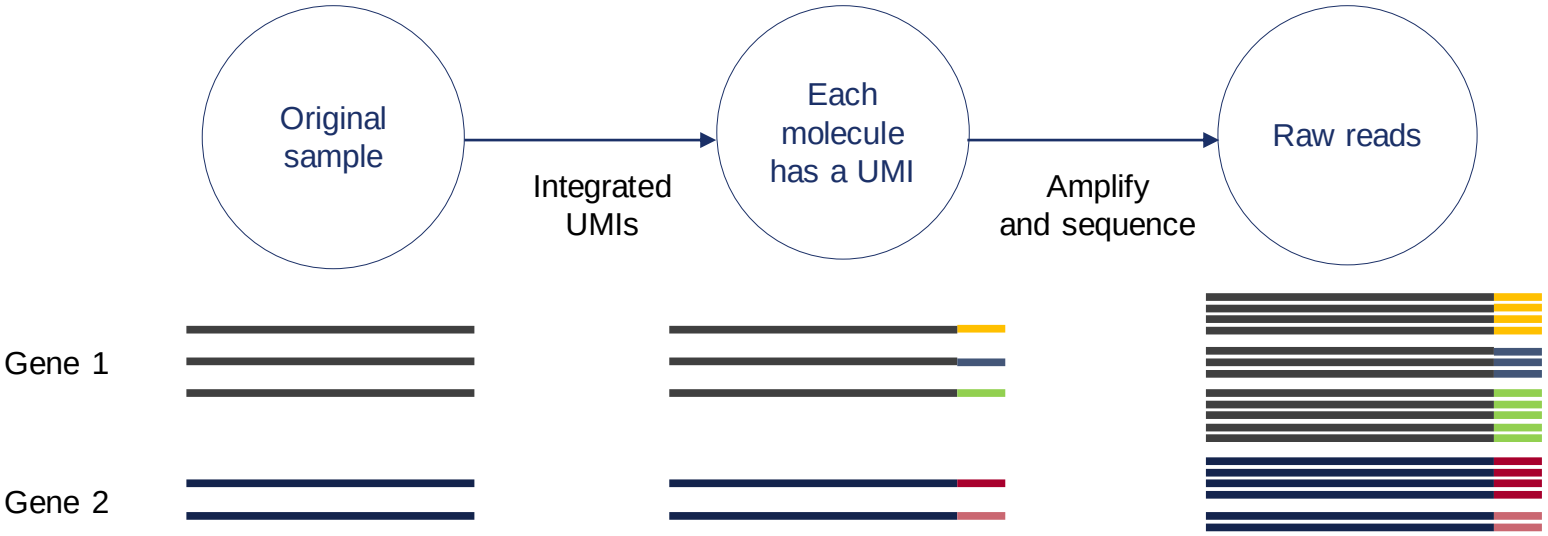


Next-generation sequencing: QIAseq miRNA and competitor 1 (C1)

- For C1, prior to sequencing, miRNA library was excised and purified from a PAGE gel
- MiSeq: 75 bp Single-Read (QIAseq miRNA) and 50 bp Single-Read (C1)

With QIAseq miRNA, increase your mapped miRNA reads (as a result of reduced bias and improved sensitivity) while reducing your workflow time

The principle of Unique Molecular Indices (UMIs)



Original sample (3:2 ratio of gene 1 to gene 2)

- Gene 1: 3 molecules
- Gene 2: 2 molecules

Interpretation of raw reads (2:1 of gene 1 to gene 2)

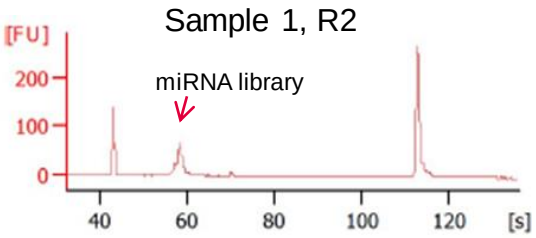
- Gene 1: 12 reads
- Gene 2: 6 reads

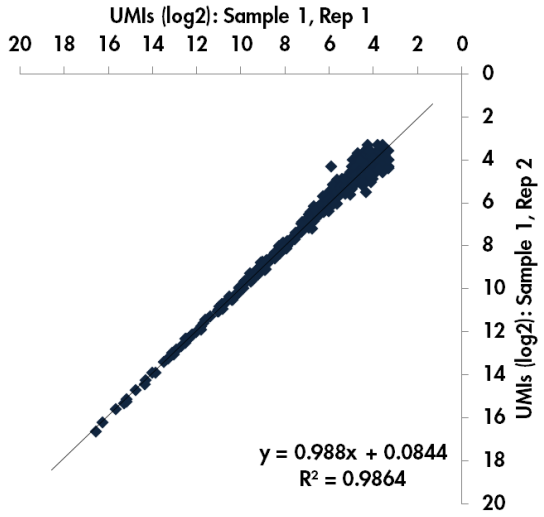
Interpretation of UMIs (3:2 ratio of gene 1 to gene 2)

- Reads are collapsed based on molecule counts
- Gene 1: 12 reads but 3 molecules are identified due to UMIs
- Gene 2: 6 reads but 2 molecules are identified due to UMIs

Quantification based on UMIs reflects quantities of original RNA molecules

Exosome samples: High mapped miRNA percentages





read set	Sample 1, Rep 1	Sample 1, Rep 2	Sample 2, Rep 1	Sample 2, Rep 2
total_reads	3,454,577	3,539,076	2,531,228	6,230,468
no_adapter_reads	321,093	276,815	345,974	562,212
too_short_reads	737,228	799,712	461,630	1,240,571
UMI_defective_reads	192,311	154,736	194,358	398,158
miRNA_Reads	1,333,379	1,424,014	913,946	2,413,667
hairpin_Reads	2,787	2,851	2,078	6,554
piRNA_Reads	29,049	30,768	23,773	62,763
rRNA_Reads	93,880	92,517	83,114	201,736
tRNA_Reads	18,248	18,566	16,370	41,872
mRNA_Reads	12,127	12,383	9,533	24,309
otherRNA_Reads	149,227	152,885	88,306	239,613
notCharacterized_Mappable	135,715	139,635	120,375	326,720
notCharacterized_notMappable	429,533	434,194	271,771	712,293
miRNA Mapping %	38.6	40.2	36.1	38.7

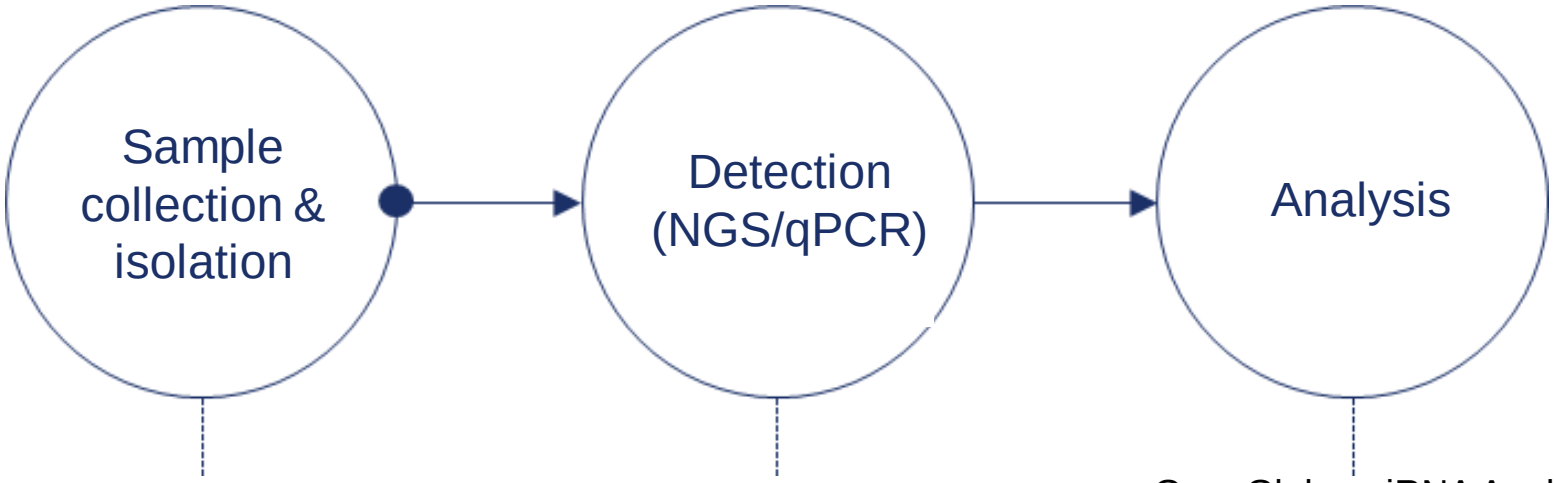
Isolation: exoRNeasy
(1 ml plasma processed)
Samples: Four total RNA samples
(2 donors, 2 replicates)
RNA input: 5 µl of RNA eluate

Outcome: High mapping percentage to miRNAs; low mapping percentage to other RNA reads (often observed with other commercial kits).

Agenda

1. miRNA introduction
2. Sample types and considerations
3. The new miRNeasy Serum/Plasma Kit
4. Quality control
5. miRNA analysis with NGS
6. Summary and questions

QIAGEN offers a complete portfolio for miRNA research



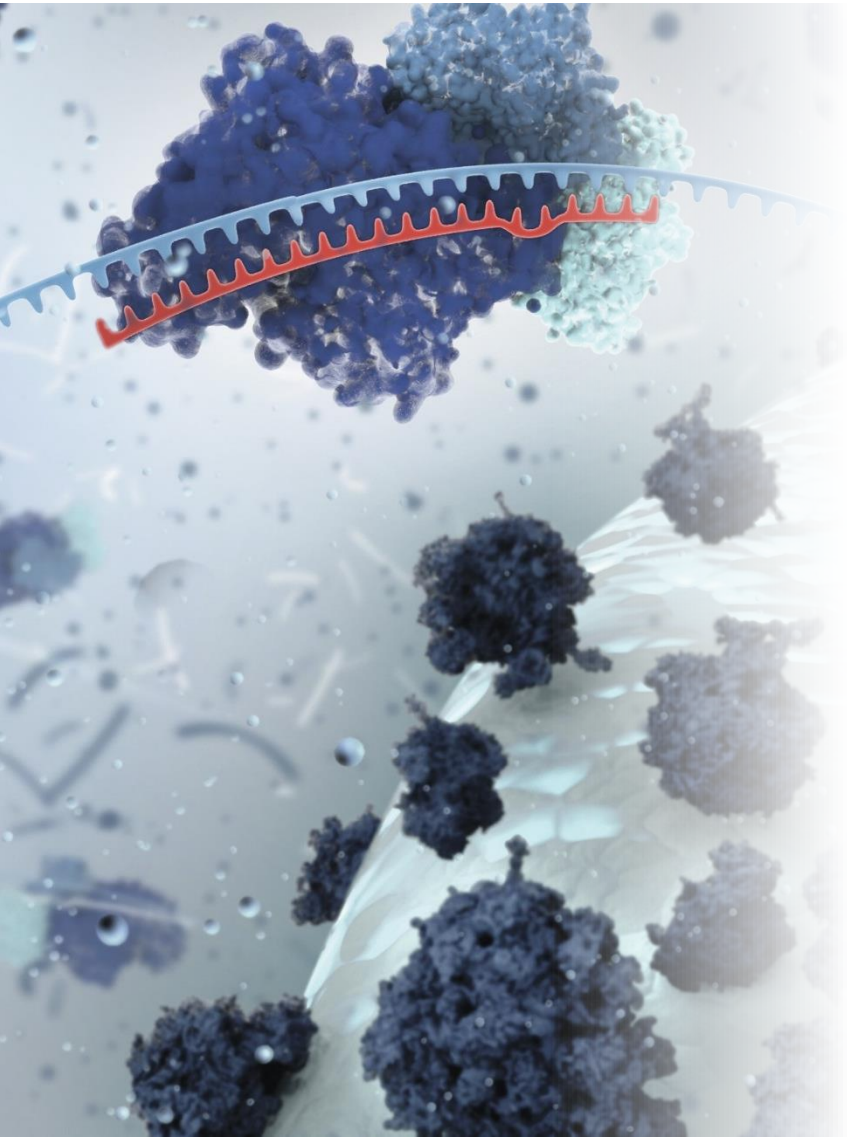
- miRNeasy Mini, Micro
- miRNeasy FFPE
- miRNeasy Serum/Plasma Advanced
- PAXgene Tissue miRNA
- PAXgene Blood miRNA

- QIAseq miRNA Library Kit
- miRCURY LNA qPCR arrays and assays

- GeneGlobe miRNA Analysis Tool
- Ingenuity Pathway Analysis

For a detailed discussion of miRNA detection join us for Part 2!

Two-part webinar series on circulating RNA biomarkers



Part 1: Advancing miRNA research – best practices for your experiment

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

Part 2: Successfully detect miRNAs using qPCR with LNA technology

- Advantages of miRNA detection using qPCR with LNA technology (locked nucleic acids)

Thank you for your attention!

Questions?

qiawebinars@qiagen.com

QIAGEN TechnicalService

Call: 1-800-426-8157 (US) / +49 2103-29-12400 (EU)

www.support.qiagen.com