



The underestimated role of rRNA removal from FFPE and liquid biopsy samples Generating consistent on-target reads for gene expression and miRNA data

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Agenda

Background

RNA-seq: FFPE and whole blood samples

miRNA-seq: Serum/plasma samples

Summary



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Summary

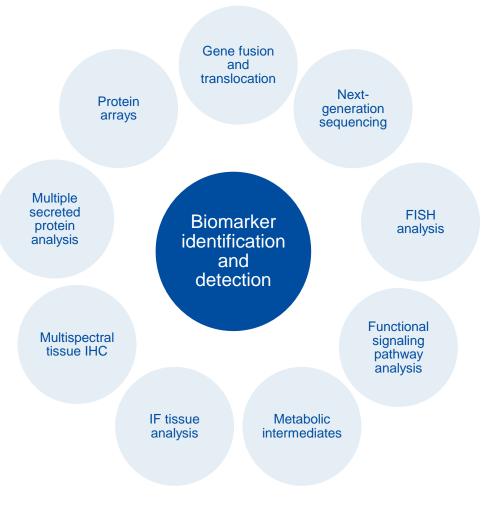


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What is a biomarker?

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention.

Characteristic(s)	Methodology
Presence of antibodies	Elisa
Abnormal BP, blood cell counts, electrolytes	Blood counts, blood pressure
Distinct histological indicators	Microscopy
Abnormal liver function markers	Biofluid assay
Presence of muscle injury protein markers	Biofluid assay
Elevated kidney marker – serum creatinine	Biofluid assay
Gene status or gene expression status	qPCR, NGS, array, etc.



Sample types typically used for biomarker studies



- Fresh/frozen cells and tissues
- FFPE tissues
- Serum/plasma
- Biofluids
- Whole blood
 - Tip: Choose sample prep kits that provide you with the flexibility to analyze all RNAs.



FFPE tissues: Classic yin and yang

The dark side of FFPE

RNA is chemically modified, cross-linked and degraded

- Time is not favorable to FFPE sample quality
- Different fixation and tissue processing methods create variability in quality

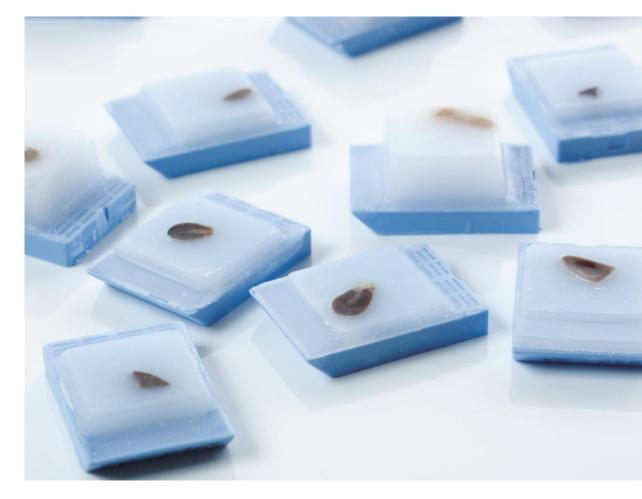
Unfavorable signal-to-noise ratio

- Fragments of RNAs
- Bacteria RNA contamination
- Perception that gene & miRNA signatures are locked away and inaccessible

The bright side of FFPE

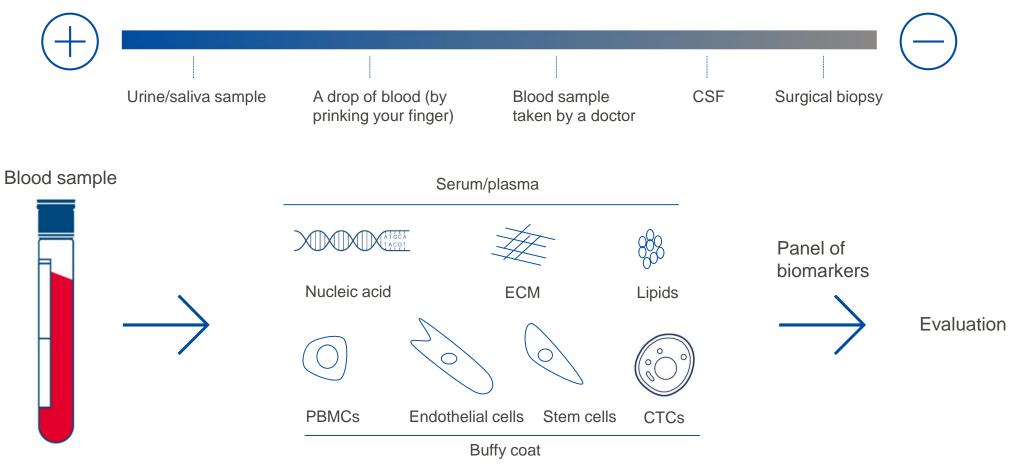
Millions of FFPE samples exist worldwide

- · Well annotated, containing clinical and demographic data
- Increased usage in basic and translational research
- · Reality is that molecular analyses are possible
 - Fragments of RNAs
 - Bacteria RNA contamination
- The challenge: Achieve a data yield comparable to fresh frozen samples.



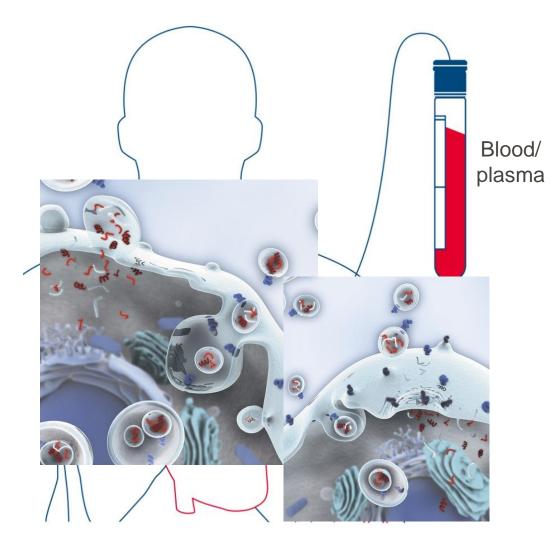
Liquid biopsies

To use as a biomarker source for diagnostics, the sample must be as easy to obtain as possible



Source: Martin, K. J. et. al. (2010) A need for basic research on fluid-based early detection biomarkers. Cancer Res. 70(13): 5203-6

Exosomes or microvesicles (MVs)



- Exosomes or MVs are ~50-200 nm small vesicles excreted by all cells
- Exosomes are found in all biofluids (e.g., blood)
- Exosomes contain stable RNA (mRNA, miRNA and other small RNAs), DNA and proteins, protected from degradation by a lipid bilayer
- Contents are specifically packaged
- · Mechanism of local and distant cellular communication



Sample preparation: A total RNA solution for every sample type

FFPE tissue Whole blood • miRNeasy FFPE Kit • PAXgene® Blood miRNA Kit • AllPrep® DNA/RNA FFPE Kit

Serum/plasma

- miRNeasy Serum/Plasma Advanced Kit
- miRNeasy Serum/Plasma Kit
- QIAamp[®] ccfDNA/RNA Kit

Exosome enrichment/isolation from serum/plasma

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

Tip: Heparin is a potent reverse transcription inhibitor. For a custom solution from QIAGEN, drop me an email.

RNA-focused next-generation sequencing (NGS)

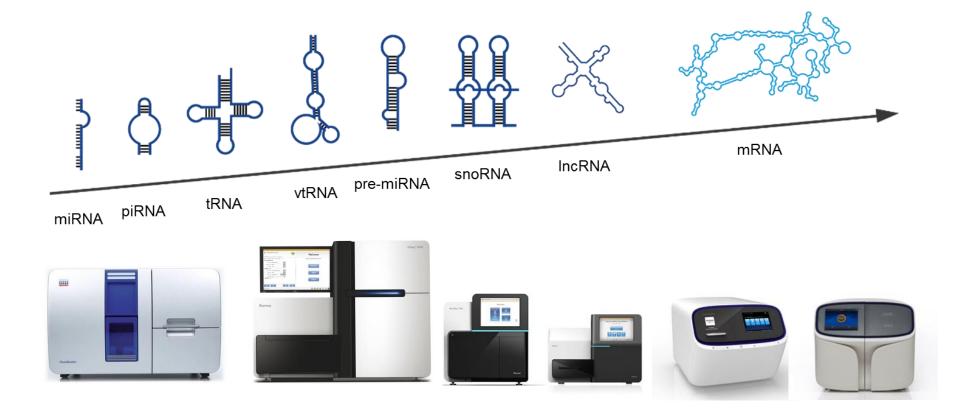
Specific applications

Specific analytes

• Gene expression

QIAGE

- Pathway analysis
- Biomarker discovery
- Allele-specific expression
- Fusion genes in cancer
- Tumor heterogeneity
- Immune repertoire
- Single-cell sequencing



All RNAs are unique, and their individual properties matter

mRNA	IncRNA	miRNA/small RNA
Coding	Non-coding, regulatory functions	Non-coding, regulatory functions
200–100,000 nt	200–100,000 nt	19–25 nt
5' cap, poly(A), splicing	5' cap, may have poly(A), splicing, complex loci	No poly(A), processed from longer precursors
~21,000 human genes	~15,000 human genes (predicted 3–100 fold of mRNA in number)	2,588 human miRNAs
Low to high expression level	Very low to moderate expression level	Very low to high expression level

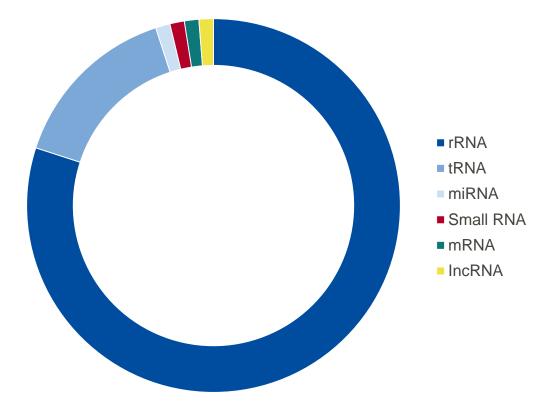
Tip: The unique properties of RNAs demand specialized library prep solutions.

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Library enrichment/depletion strategies: Whole transcriptome NGS

Enrichment or depletion is necessary to maximize reads from the RNAs of interest

- Typical RNA composition in a cell: >80% ribosomal RNA (in blood cells, globin is also a major contaminant)
- Highly abundant transcripts consume a lot of reads
- Enrichment or depletion is used to obtain more reads from the RNAs of interest, such as:
 - mRNA
 - IncRNA



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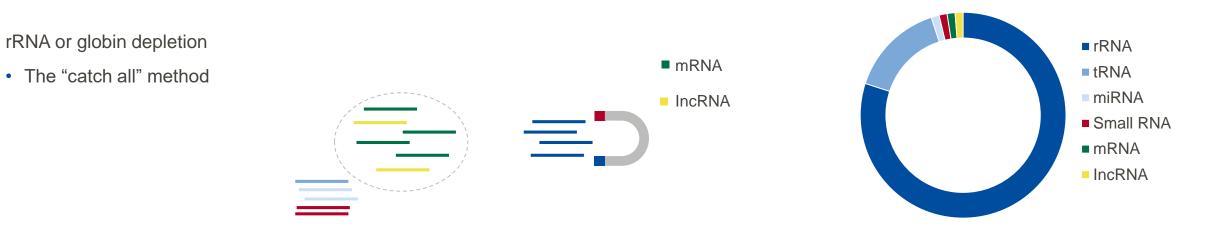
Library enrichment/depletion strategies: Whole transcriptome NGS

Enrichment/depletion strategies

Poly(A) enrichment

- Not useful for fragmented samples
- Not useful for prokaryotic samples





Strategy selection depends on the objective of your whole transcriptome sequencing.

In miRNA-seq, hY4 Y RNA can be a major contaminant

Read set	hY4 not blocked (two re	hY4 not blocked (two replicate libraries)		
Total_reads	2,266,831	2,715,234		
No_adapter_reads	98,237	120,162		
Too_short_reads	346,580	383,548		
UMI_defective_reads	44,259	50,209		
miRNA_reads	596,675	680,215		
Hairpin_reads	1,216	1,468		
piRNA_reads	11,819	14,441		
rRNA_reads	39,078	47,098		
tRNA_reads	8,556	10,628		
mRNA_reads	5,251	6,163		
OtherRNA_reads	833,042	1,071,052		
NotCharacterized_mappable	72,481	84,476		
NotCharacterized_notmappable	209,637	245,774		
miRNA mapping %	26.3	25.1		
OtherRNA_reads (hY4 mapped here) %	36.7	39.4		

When you do not block hY4, mapping to "OtherRNA_reads" increases from ~2.5% to ~40%. In addition, miRNA mapping decreases from ~40% to 25%.



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RNA-seq: FFPE and whole blood samples

miRNA-seq: FFPE and serum/plasma samples

Summary



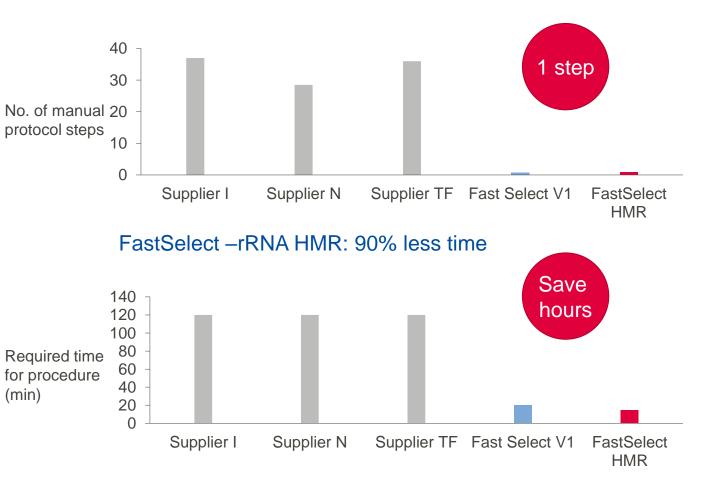
QIAseq[®] FastSelect –rRNA HMR Kit

Removes rRNA in a single step requiring only 14 minutes

(min)

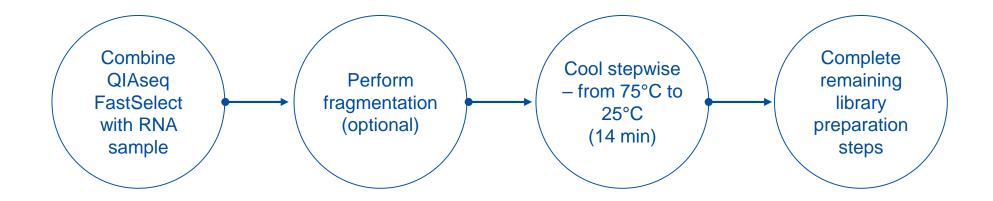
- Problem: rRNA is a significant NGS contaminant
 - 80–90% of RNA in samples
- Existing depletions: Slow, tedious
 - Number of steps: Typically 29–37
 - Time required: 2+ h
- QIAseq FastSelect: Revolutionary
 - Number of steps: One
 - Time required: 14 min 0
 - Robustness: >99% rRNA removal 0
 - Compatibility: Any RNA library prep kit

FastSelect –rRNA HMR: Only one step



FastSelect –rRNA HMR plus –Globin

Thirty percent faster than FastSelect V1, now in a single-tube



FastSelect -- rRNA HMR plus -- Globin

How does FastSelect -rRNA HMR plus -Globin work?

- Inhibits reverse transcription of specific targets
- Removes cytoplasmic and mitochondrial rRNA and/or globin mRNA

Species covered in a single-tube:

- Human, mouse, rat (HMR) and other mammalian species
- HMR removes 95–99% rRNA from cow, horse, sheep and hamster samples
- HMR removes 80–90% rRNA from dog, chicken, rabbit, pig and monkey samples

RNA compatibility:

- Total RNA: Use FastSelect –rRNA HMR (include –Globin if working with whole blood)
- Poly(A) enriched RNA: Use FastSelect –Globin if working with whole blood

Sample compatibility:

• Cell lines, tissues (fresh/frozen), FFPE tissues, blood and biofluids

Total RNA input:

• 1 ng – 1 μg

Tested RNA library prep kit compatibility:

- QIAseq Stranded Total RNA Lib Kit (QIAGEN), TruSeq[®] Stranded (Illumina[®]), NEBNext[®] Ultra II Directional RNA Library Prep Kit (NEB[®]), KAPA[®] RNA HyperPrep Kit (Roche Group)
- FastSelect is compatible with most RNA library prep kits

FastSelect –rRNA HMR: Robust performance with FFPE samples

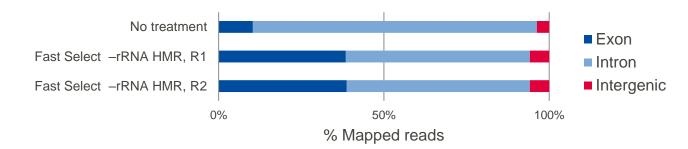
Experimental overview

QIAGE

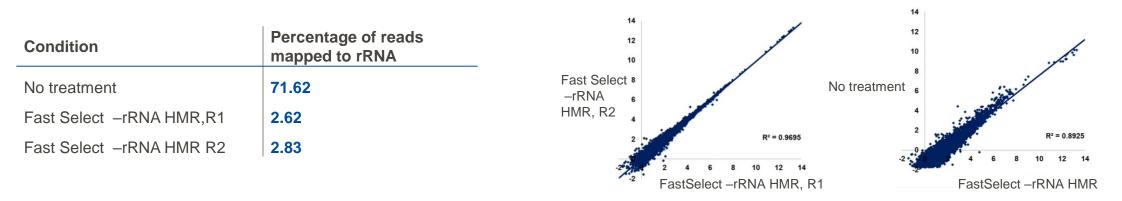
- Sample: 225 ng, Fusion RNA Positive Control (Horizon™)
- Depletion: No depletion, FastSelect
- Library prep: QIAseq Stranded Lib Kit
- Sequencing: NextSeq 550 (2 x 75 bp)
- Mapping: CLC Genomics Workbench

Substantial rRNA removal

Increased/consistent exon mapping



Resulting gene expression profiles strongly correlate



FastSelect efficiently removes rRNA from FFPE samples, resulting in increased mapping to exons. Gene expression values from FastSelect treatments are highly correlative, without off-target effects (Log2 RPKM > 0.3).

QIAseq FastSelect: Robust performance with FFPE samples

Experimental overview

QIAGE

Sample: 100 ng normal and cancer lung FFPE
 Sequencing: NextSeq 550 (2 x 75 bp)

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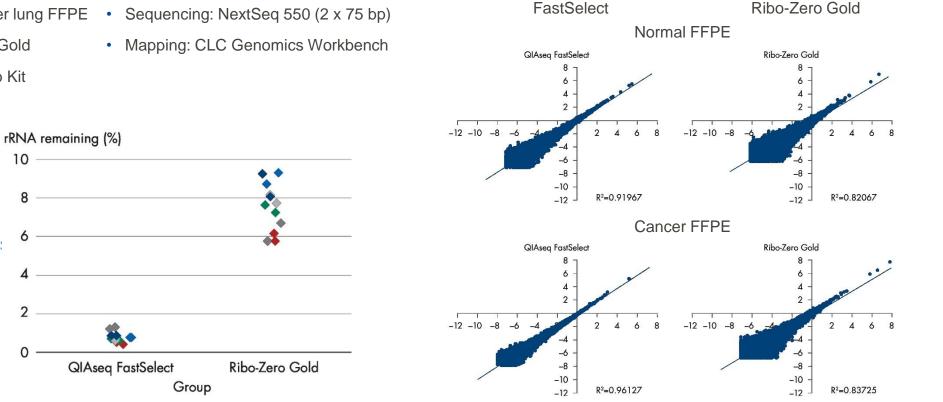
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- Depletion: FastSelect, Ribo-Zero Gold
- Library prep: QIAseq Stranded Lib Kit

FastSelect robustly remove:

rRNA from FFPE Samples



FastSelect robustly removes rRNA and enables high reproducibility of experiments. Ribo-Zero was not as effective with FFPE samples and required more amplification, suggesting loss of sample material.

Replicate samples: Gene expression

FastSelect –rRNA HMR plus –Globin: Robust removal of rRNA and Globin

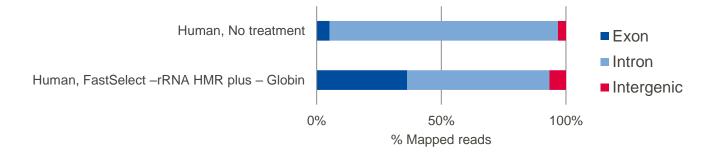
Experimental overview

QIAGE

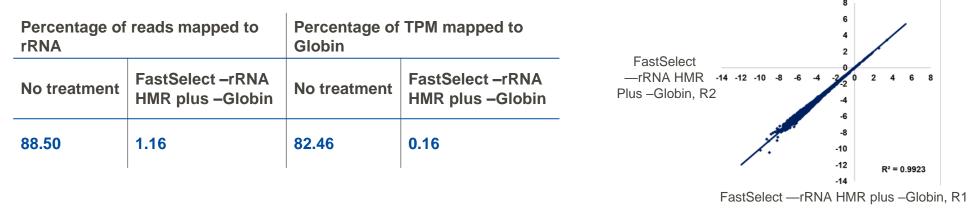
- · Sample: 100 ng human whole blood total RNA
- Depletion: FastSelect -rRNA HMR plus -Globin
- Library prep: QIAseq Stranded Lib Kit
- Sequencing: NextSeq 550 (2 x 75 bp)
- Mapping: CLC Genomics Workbench

Substantial removal of rRNA and Globin

Increased exon mapping



Resulting gene expression profiles strongly correlate



FastSelect efficiently removes rRNA and Globin, resulting in an increased percentage of reads mapped to exons. Gene expression values from FastSelect-treated samples are highly correlative (Log2 RPKM > 0.3).

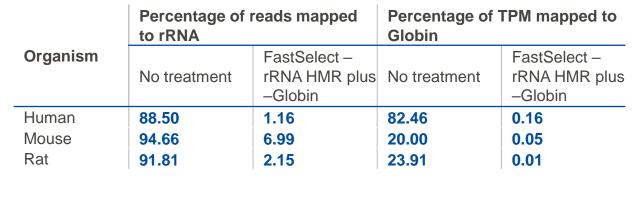
FastSelect -rRNA HMR plus -Globin: Robust removal of rRNA and Globin (cont.)

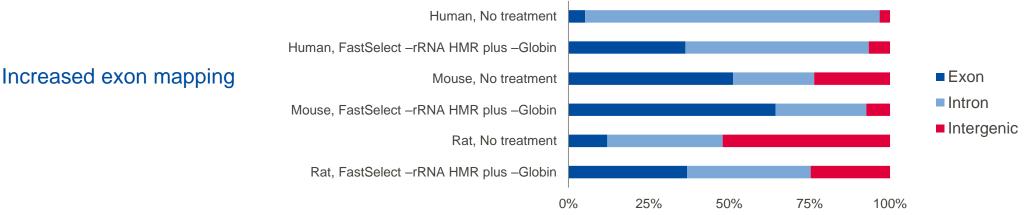
Experimental overview

QIAGEN

- Total RNA: Human, mouse, and rat whole blood
- Depletion: FastSelect –rRNA HMR plus –Globin
- Library prep: QIAseq Stranded Lib Kit
- Sequencing: NextSeq 550 (2 x 75 bp)
- Mapping: CLC Genomics Workbench

Substantial removal of rRNA and Globin





FastSelect efficiently removes rRNA and Globin, resulting in an increased percentage of reads mapped to exons. Gene expression values from FastSelect-treated samples are highly correlative (Log2 RPKM > 0.3).

FastSelect –Globin: Robust removal of Globin from mRNA-enriched samples

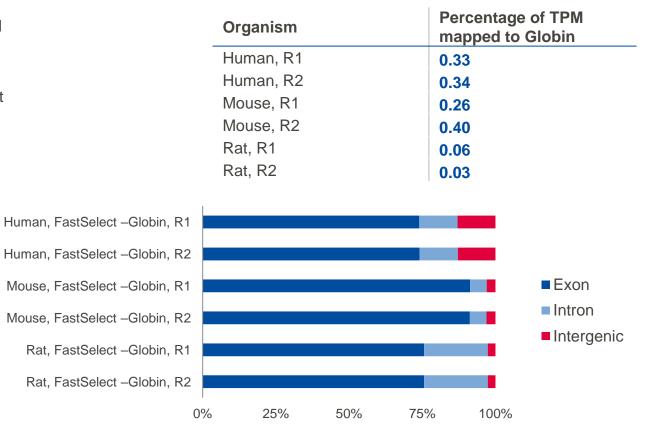
Experimental overview

QIAGEN

- Total RNA: Human, mouse, and rat whole blood
- Depletion: FastSelect –Globin
- Library prep: QIAseq Stranded mRNA Select Kit
- Sequencing: NextSeq 550 (2 x 75 bp)
- Mapping: CLC Genomics Workbench

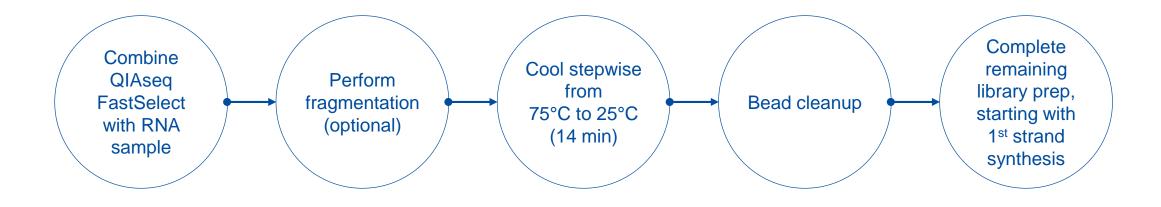
Consistently high exon mapping

Substantial removal of rRNA and globin



FastSelect efficiently removes Globin from mRNA-enriched samples. Compared to total RNA library preps, mRNAenriched libraries exhibit a high percentage of reads mapped to exons.

FastSelect –5S/16S/23S: An overview



FastSelect –5S/16S/23S: An overview

What is FastSelect -5S/16S/23S?

 Fragmentation and pan-bacterial (5S/16S/23S) rRNA depletion module

Number of reactions:

• 24, 96 and 384

How does it work?

• Inhibits reverse transcription of its specific targets

Coverage:

- Designed to block community level cDNA synthesis of 5S, 16S and 23S rRNA
- Designed against SILVA 16S sequences (nearly 600,000 unique entries), SILVA 23S sequences (nearly unique 170,000 entries) and 5S rRNA Database (over 7,200 unique entries)
- Theoretically blocks >95% cDNA synthesis of all 5S, 16S and 23S rRNA sequences
 - In practice, results will vary, based on the exact composition of the sample

Total RNA input:

• 20 ng to 1 µg

Tested RNA library prep kit compatibility:

- QIAseq Stranded Total RNA Lib Kit (QIAGEN Group), TruSeq[®] Stranded (Illumina[®], Inc), NEBNext[®] Ultra II Directional (New England Biolabs, Inc)
- FastSelect is compatible with most RNA library prep kits

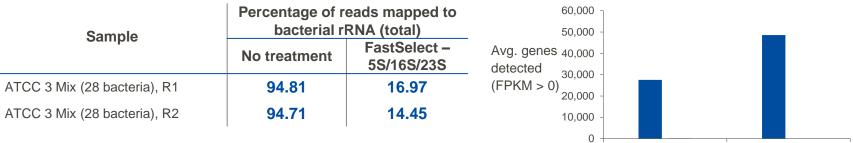
FastSelect –5S/16S/23S: Robust rRNA removal from bacterial communities

Experimental overview

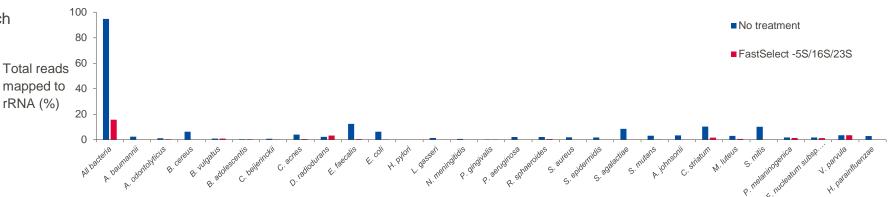
QIAGE

- Sample: 100 ng, 20 Strain Even Mix Whole Cell Material (ATCC) + Skin Microbiome Whole Cell Mix (ATCC) + Oral Microbiome Whole Cell Mix (ATCC)
- Depletion: No depletion, FastSelect
- Library prep: QIAseq Stranded
- Sequencing: NextSeq 550
- Mapping: CLC Genomics Workbench

FastSelect substantially removes rRNA



FastSelect robustly depletes rRNA (individual species)



FastSelect efficiently removes rRNA, freeing up substantial read budget. In turn, this read budget enables a dramatic increase in the number of genes detected.

FastSelect increases detected genes

No treatment

FastSelect -5S/16S/23S

FastSelect –rRNA HMR seamlessly integrates with FastSelect –5S/16S/23S

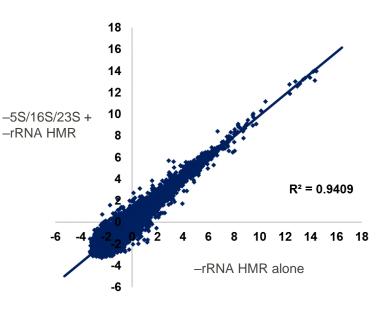
Experimental overview

- Sample: 100 ng, different ratios of Universal Human (H) + Gut (G) RNA
- Depletion: No depletion, FastSelect –5S/16S/23S + FastSelect –rRNA HMR
- Library prep: QIAseq Stranded Total RNA Lib Kit
- Sequencing: NextSeq 550 (2 x 75 bp)
- Mapping: CLC Genomics Workbench

FastSelect –rRNA HMR + –5S/16S/23S robustly removes rRNA and matches theoretical calculations*

Sample Percentage of reads mapped to human + bacterial rRNA (total) (Sample ratio (human [H] No FastSelect **FastSelect** and gut [G]) treatment (theory) (actual) 0 H : 100 G 9.87 92.78 9.87 1 H : 99 G 92.74 9.78 9.76 10 H : 90 G 92.37 9.01 8.91 25 H : 75 G 92.30 7.73 8.84 50 H : 50 G 91.88 5.58 6.64 75 H : 25 G 91.84 3.44 3.49 90 H : 10 G 90.90 2.15 2.39 99 H : 1 G 1.31 90.97 1.38 100 H : 0 G 90.99 1.29 1.29

FastSelect –rRNA HMR + –5S/16S/23S does not negatively impact human GenEx



* Theoretical calculations based on 100% gut and 100% human values

FastSelect –rRNA HMR + –5S/16S/23S efficiently removes rRNA from combined human + bacteria samples. FastSelect –5S/16S/23S does not negatively impact human gene expression, because in the presence of –5S/16S/23S human gene expression patterns remain the same.



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RNA-seq: FFPE and whole blood samples

miRNA-seq: Serum/plasma samples

Summary



QIAseq miRNA Library Kit: miRNA-focused library prep kit

Distinguishing features of the QIAseq miRNA Library Kit

Gel- and adapter dimer-free workflow from 1–500 ng of total RNA Naturally eliminates:

- Adapter dimer
- rRNA
- hY4 Y RNA

Integrated unique molecular index (UMI) technology

Compatible with Illumina® and Thermo Fisher Scientific® sequencers

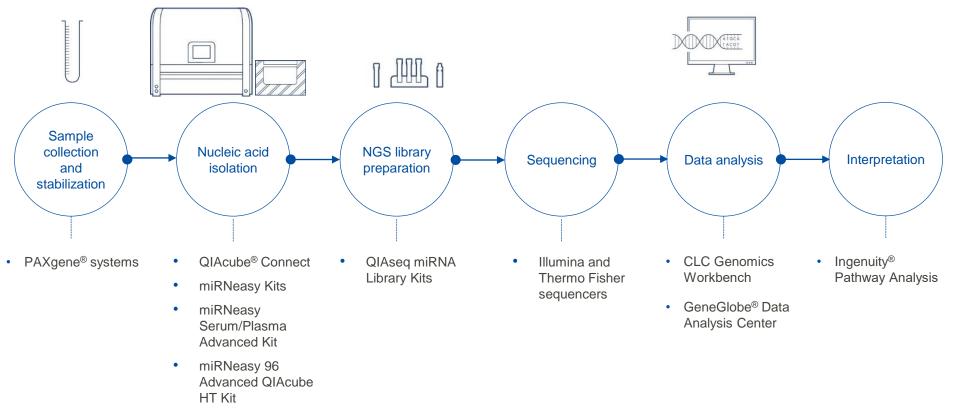
When compared to other kits, the QIAseq Kit ranked*:

- · Highest in mapped reads from serum and plasma
- Highest in mapped reads from brain tissue
- The most efficient for biofluids and tissue



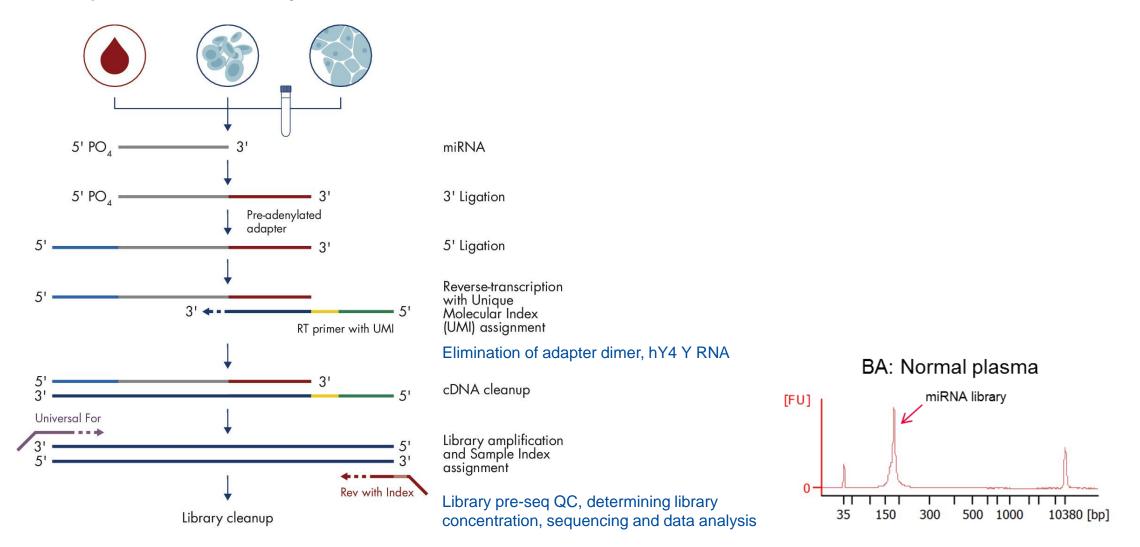
*Source: Coenen-Stass A.M.L. et al. (2018) Evaluation of methodologies for microRNA biomarker detection by next generation sequencing, RNA Biology, 15:8, 1133–1145

Sample to Insight solutions for miRNA sequencing



exoRNeasy Kits

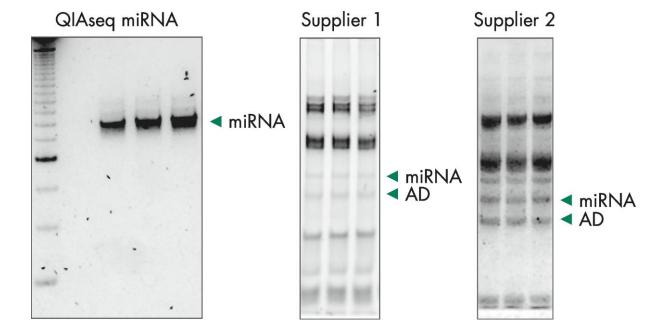
QIAseq miRNA: Library construction



The underestimated role of rRNA removal from FFPE and liquid biopsy samples 32

QIAseq miRNA: Natural elimination of rRNA

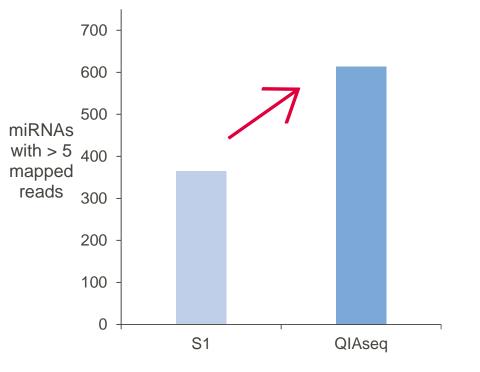
PAGE gel after standard library prep protocol



RNA input: 100 ng (QIAseq miRNA), 1 µg (Supplier 1), 100 ng (Supplier 2)

QIAseq miRNA gives you a robust, specific miRNA library with negligible background.

2x mapped miRNAs with QIAseq

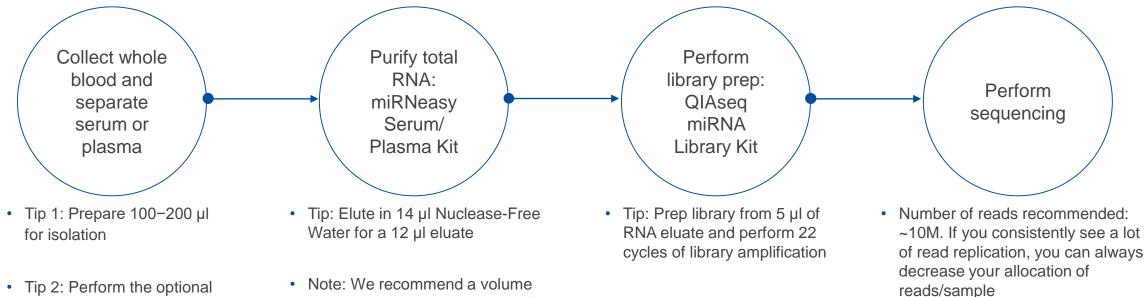


QIAseq miRNA eliminates hY4 Y RNA

Read set	hY4 not blocked	hY4 not blocked (two replicate libraries)		o replicate libraries)
Total_reads	2,266,831	2,715,234	2,293,207	3,037,150
No_adapter_reads	98,237	120,162	135,763	189,849
Too_short_reads	346,580	383,548	620,065	814,789
UMI_defective_reads	44,259	50,209	59,749	80,263
miRNA_reads	596,675	680,215	908,267	1,211,195
Hairpin_reads	1,216	1,468	1,870	2,351
piRNA_reads	11,819	14,441	19,091	24,937
rRNA_reads	39,078	47,098	57,720	76,580
tRNA_reads	8,556	10,628	12,868	16,746
mRNA_reads	5,251	6,163	8,291	10,723
OtherRNA_reads	833,042	1,071,052	60,577	81,572
notCharacterized_mappable	72,481	84,476	96,966	126,366
notCharacterized_notmappable	209,637	245,774	311,980	401,779
miRNA mapping %	26.3	25.1	39.6	39.9
OtherRNA_reads	36.7	39.4	2.6	2.7

When you block hY4, mapping to "OtherRNA_Reads" decreases from ~40% to ~2.5%. In addition, miRNA mapping increases from ~25% to 40%.

Workflow: Total RNA from serum and plasma



 Tip 2: Perform the optional spins to remove cellular nucleic acids attached to cell debris Note: We recommend a volume equivalents approach instead of measuring RNA concentration as serum/plasma samples show low RNA concentration readings due to lack of rRNA.

• Note: hY4 Y RNA is blocked

The underestimated role of rRNA removal from FFPE and liquid biopsy samples 35

Serum and plasma: High mapped miRNA percentages

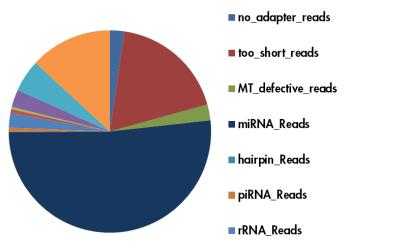
miRNeasy Serum/Plasma Kit: 200 µl input

Total RNA input: 5 µl RNA eluate (80 µl of serum equivalents)

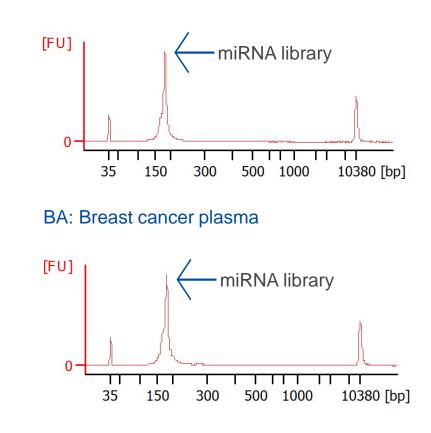
- Normal (N) plasma (n = 3)
- Breast cancer plasma (n = 3)

Sequencing: NextSeq, 75 bp Single Read

miRNA mapping %: 52 (N1), 54 (N2), 47 (N3), 49 (BC1), 41 (BC2), 57 (BC3)



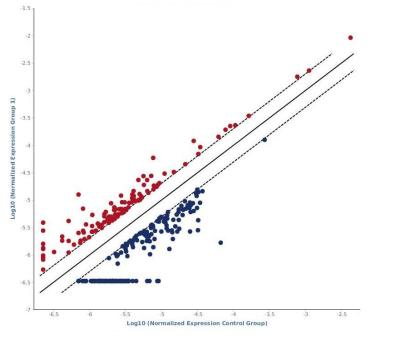
BA: Normal plasma



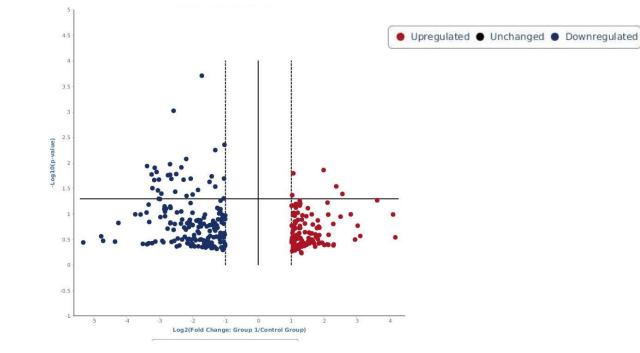
The QIAseq miRNA workflow prepares robust libraries from plasma enabling a high percentage of miRNA reads without gel excision.

QIAseq miRNA: Optimized quantification of miRNAs from serum and plasma





Volcano plot (fold-regulation vs. p-value)



Upregulated miRNAs = 123 (6 significantly)

miR-520g-3p: Associated with important prognostic factors in breast cancer patients

Downregulated miRNAs = 179 (31 significantly)

Use the GeneGlobe Data Analysis Center to easily identify differentially expressed miRNAs.

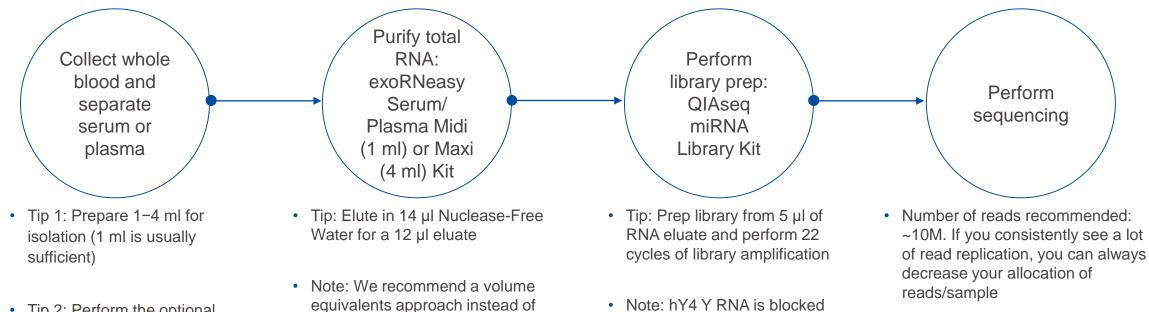
Workflow: Exosomal total RNA from serum and plasma

measuring RNA concentration as

serum/plasma samples show low

lack of rRNA.

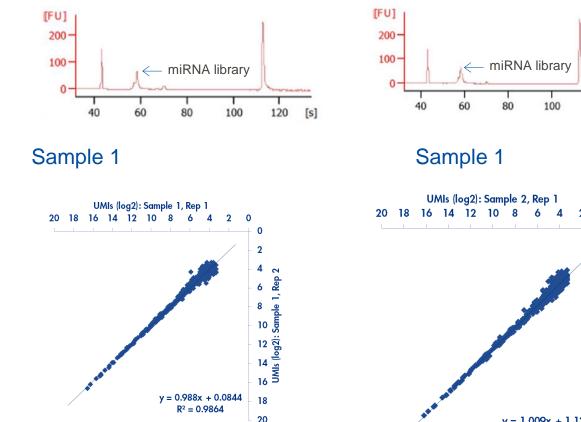
RNA concentration readings due to



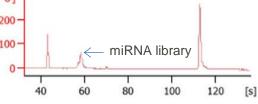
 Tip 2: Perform the optional spins to remove cellular nucleic acids attached to cell debris

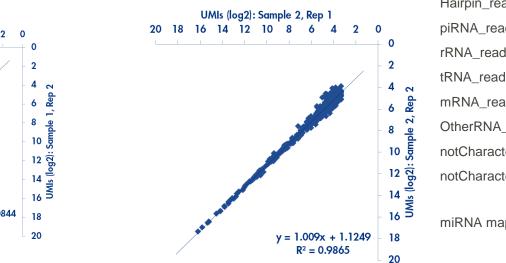
Exosome samples: High mapped miRNA percentages

Sample 1, R1



Sample 1, R2

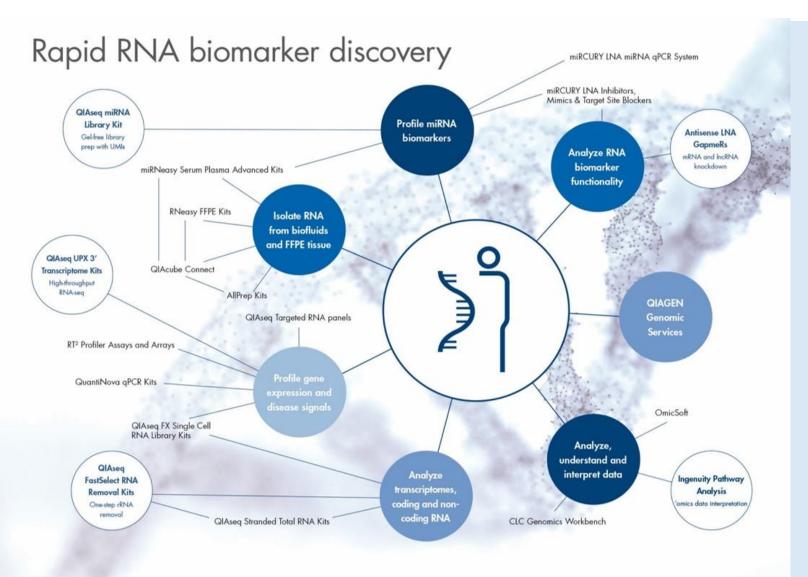




Read set	Sample 1, R1	Sample 1, R2	Sample 2, R1	Sample 2, R2
Total_reads	3,454,577	3,539,076	2,531,228	6,230,468
No_adapter_reads	321,093	276,815	345,974	562,212
Too_short_reads	737,228	799,712	461,630	1,240,571
UMI_defective_reads	192,311	154,736	194,358	398,158
miRNA_reads	1,333,379	1,424,014	913,946	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

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





A comprehensive portfolio for RNA biomarker discovery

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miRNA-seq: Serum/plasma samples

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RNA-seq and miRNA-seq challenges



FFPE and liquid biopsy samples are imperative for basic and translational research, but are often difficult to work with

Various contaminating RNAs can plague the library prep step

 rRNA, globin, hY4 Y RNA and adapter dimers

The new FastSelect kits



FastSelect-rRNA HMR and -Globin:

Removes cytoplasmic and mitochondrial rRNA and/or globin mRNA by inhibiting reverse transcription of specific targets

• Covers human, mouse, rat (HMR) and other mammalian species

FastSelect–5S/16S/23S: Fragmentation and pan-bacterial (5S/16S/23S) rRNA depletion module which also works by inhibiting reverse transcription of specific targets

 Blocks community level cDNA synthesis of 5S, 16S and 23S rRNA

QIAseq miRNA



An miRNA-focused library prep kit

- Naturally removes rRNA, hY4 Y RNA and adapter dimers
- Gel-free workflow from 1 ng to 500 ng of total RNA



QIAseq FastSelect: An unparalleled unwanted RNA removal solution





Thank you for attending.

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