Long Non-coding RNAs (lncRNAs) and microRNAs as Liquid biopsy biomarkers

Samuel J. Rulli, Jr.
Global Product Manager
Biological Research Content

Samuel.rulli@qiagen.com
Long non-coding RNA (IncRNA)

- Introduction to long-noncoding RNA
  - What is IncRNA?

- IncRNA and liquid biopsy
  - How IncRNAs can be part of a liquid biopsy program

- Integrating QIAGEN RT² IncRNA PCR Arrays into research
  - Application: IncRNA from serum & plasma

- Introduction to microRNA
  - What is microRNA?

- 3 step process to microRNA biomarker discovery
  - Application: microRNAs from blood for liver toxicity

- Summary
Long non-coding RNA (IncRNA)

- Long non-coding RNAs (long ncRNAs, IncRNA) are non-protein coding transcripts longer than 200 nucleotides.

- Do not contain an open reading frame – not translated using standard codons (however some evidence in yeast of alternative reading frames?)

- May contain a Poly-A tail like mRNA or may be absent

- IncRNA expression is generally lower than mRNA and sensitive method such as qPCR are needed for detection & quantitation

- The important central roles of IncRNA in biology and pathology are being demonstrated by many research work.
Functions of IncRNA

IncRNAs can be categorized into subgroups according to where they are found relative to nearby protein-coding genes:

- intergenic,
- exonic,
- intronic,
- Overlapping

(A) Proportion of IncRNA subgroups.
(B) Location of each type of IncRNA.

Lee and Kikyo Cell & Bioscience 2012 2:37
Proposed functions of IncRNA

1. Negatively affect expression
2. Positively affect expression
3. Hybridize to the pre-mRNA resulting in an alternatively spliced transcript
4. Hybridization of the sense and antisense transcripts allow Dicer to generate endogenous siRNAs
5. Binding to miRNA results in miRNA function silencing
6. The complex of IncRNA and specific protein partners can modulate the activity of the protein
7. Involved in structural and organizational roles of the cell
8. Alters the protein localizes in the cell
9. Affects epigenetic processes
10. Be processed to the small RNAs
A liquid biopsy is a liquid biomarker that can be isolated from body fluids, such as blood, saliva, urine, ascites, or pleural effusion. Like a tissue biopsy, it is a representative of the tissue from which it has spread.

Liquid biopsies have become more clinically useful in recent years due to the ability to pair tests on circulating tumor cells with genomic tests.

Tumors shed both intact cells (resulting in circulating tumor cells) as well as cellular components, such as nucleic acids (resulting in cell-free DNA or RNA).

**Liquid biopsies**

- **CTCs** (circulating tumor cells)
  - Cancer cells released from primary tumor mass into the bloodstream

- **ctNA** (circulating tumor nucleic acids)
  - ctDNA (circulating tumor DNA), miRNAs, mRNA, & long non-coding RNA

- **Exosomes**
  - Small membrane-derived vesicles (40–100 nm) contain various molecules such as signal proteins, microRNAs, mRNAs, lipids, and exoDNA.

- **CTC**
- **ctNA, mainly ctDNA**
- **Exosome vesicle, exoDNA, miRNA, and IncRNA**

**Samples:** blood, serum/plasma, urine, CSF, saliva

Exosomes: small membrane vesicles (30–100 nm), secreted by most cell types into the bloodstream.

- **Exosomes play a central role in cell-to-cell communication.**

- **The majority of DNA associated with tumor exosomes is double-stranded, representing whole genomic DNA.**

- **Biological molecules (protein, RNA, and miRNA) contained in exosomes are well protected by a lipid bilayer membrane that confers a high degree of stability.**

**Functional biomolecules:**
- DNA fragments (exosomal DNA, exoDNA)
- Proteins and/or peptides
- mRNA
- microRNA (miRNA)
- Lipids

IncRNAs as biomarkers from urine

- Comparison study of IncRNAs
- Human prostate cell line vs. normal
- Prostate adenocarcinoma vs normal
- Urine from prostate cancer vs urine from normal or benign cancers
- Identified 6 IncRNAs in prostate adenocarcinoma vs. normal
- Same 6 IncRNAs found in urine
- New biomarker?
IncRNA identification & quantification approaches

- RNA-seq (Whole transcriptome sequencing)
  - Discover new RNAs and splicing variants

- Microarrays
  - Use data analysis approaches to identify IncRNAs
  - Retrospective studies of microarray data

- Real-time PCR based approaches
  - Sensitive and quantitative for low expressing RNAs and small gene changes
Typical IncRNA workflow and opportunities

**RNA-seq Discovery Workflow: What RNAs are present?**

1. **Samples**
2. **Isolation**
3. **RNA-Seq**
4. **qPCR verification**
5. **Data Interpretation**

- Individual RT² IncRNA qPCR Assays and Custom RT² IncRNA PCR Arrays for verification of data and to expand observations on more samples.

**Hypothesis Driven Workflow: Are IncRNAs doing something?**

1. **Samples**
2. **Isolation**
3. **qPCR**
4. **Data Interpretation**

- **qPCR Discovery**
  - RT² IncRNA PCR Arrays by disease, or pathway

- **qPCR Verification of known IncRNAs**
  - RT² IncRNA qPCR Assays or Custom RT² IncRNA PCR Arrays
Real-time PCR quantification of IncRNAs

- Has been the gold standard for gene quantification – and for reliably seeing small, but significant changes

- Is the method of choice to confirm next-generation sequencing and microarray results

- Simple and easy to carry out, able to do several replicates and build statistical sets

- Able to use pre-amplification and WTA strategies (FFPE samples and single cell analysis). This is a big advantage for detecting something that you are looking for.
Lung cancer is the leading cause of cancer death and the second most common cancer.

About 224,210 new cases will be diagnosed in 2014 (US) and about 159,260 deaths are expected to occur in 2014 (US).

Non-Small Cell Lung Cancer (NSCLC) is the most common type of lung cancer (about 85%).

Only recommended screening test for lung cancer is low-dose computed tomography.

Limited work has been done on serum/plasma IncRNA.

The role of IncRNA in Lung cancer have drawn attention from researchers. IncRNAs such as MALAT1, BCYRN1, LINC00970, and H19 in lung cancer have been studied.


Ref: Yong Yang, etc. The Noncoding RNA Expression Profile and the Effect of IncRNA AK126698 on Cisplatin Resistance in Non-Small-Cell Lung Cancer Cell. PLOS One, 2013.
Materials and Methods

- **Samples**
  - Control Group: Normal healthy donor samples
  - Disease group: NSCLC patient samples

- Total RNA from serum sample – (isolated with miRNeasy serum/plasma kit)

- Reverse transcription with RT2 PreAMP cDNA Kit

- IncRNA PreAMP primer mix for Cancer PathwayFinder
- RT2 IncRNA PCR Array-Human Cancer PathwayFinder (84 cancer related IncRNA assay with controls in 96-well plate).

- Master mix: RT2 SYBR Green ROX™ qPCR Mastermix

- qPCR cyclers: ABI 7900HT

- Data analysis: GeneGlobe Data Analysis Center
Sample to Insight

Protocol for RT2 IncRNA PCR Arrays with PreAmplification

- Integrated smoothly with QIAGEN’s from sample to result workflow.

- Use QIAGEN sample prep for RNA purification. (recommend miRNesay kits for new projects)

- Use RT² First strand PreAmp kit for cDNA conversion
  - Integrated DNAase step
  - Proprietary Spike in RNA
  - Priming both oligo-dT and random hexamers

- Pre-Amplification for 10 cycles

- Use RT² SYBR Green master mix for qPCR with our IncRNA products.

- Go to QIAGEN’s Data Analysis Center for analyzing IncRNA expression data.
QIAGEN built an in house database which is now part of GeneGlobe with the goal to be the authoritative lncRNA databases containing over 28,000 human and 16,000 mouse lncRNA targets.

RT² IncRNA assays are in silico designed and laboratory-verified for qPCR performance - high specificity, amplification efficiency and sensitivity.

Using this database of IncRNA qPCR Assays, QIAGEN bioinformatics scientists have generated RT² IncRNA PCR arrays to facilitate comparative discovery experiments in cancer and other research fields.

Currently cover human Gencode 19, mouse Gencode M2, RefSeq Release 65

Available as ready-to-use qPCR Assay, or ready to use PCR plate (PCR Array)

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of qPCR assays designed (custom designs not included)</th>
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<tr>
<td>Mouse</td>
<td>27425</td>
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<tr>
<td>Rat</td>
<td>8510</td>
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</table>
## Formats of RT2 IncRNA PCR Arrays

### 96-well format

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<table>
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<tr>
<th></th>
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<td>PPC</td>
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<td>PPC</td>
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</table>

### 384-well format

96 x 4

- **Housekeeping genes**
- **Genomic DNA control**
- **Reverse transcription controls**
- **Positive PCR controls**

**100 ring-disk**

96 genes - 1 sample/plate
RT2 IncRNA PCR Array layout – a complete experiment each time

84 pathway-specific genes of interest

5 “REFERENCE” genes (includes 3 mRNAs and 2 lncRNAs)

Genomic DNA contamination control (GDC)

Reverse transcription controls (RTC), n=3

Positive PCR controls (PPC), n=3

Patented controls (RTC, GDC, PPC)
Cancers:
- **Bladder Cancer:** BLACAT1, H19, MALAT1, MEG3, SNHG16, TERC, TUG1, UCA1.
- **Breast Cancer:** BCAR4, GASS, H19, HIF1A-AS2, HOTAIR, JADRR, LSINCT5, MR31HG, MRPL23-AS1, TRERNA1, UCA1, XIST, ZFAS1.
- **Cervical Cancer:** CBR3-AS1, HOTAIR.
- **Colorectal Cancer:** CCAT1, CCAT2, CRNDE, H19, HOTAIR, MALAT1, PCAT1, PVT1, SNHG16, TUSC7.
- **Endometrial Cancer:** H19, HOTAIR.
- **Esophageal Squamous Cell Carcinoma:** CBR3-AS1, HOTAIR.
- **Gallbladder Cancer:** MALAT1.
- **Gastric Cancer:** CCAT1, CDK92B-AS1, GACAT1, H19, HOTAIR, HULC, LINC00152, LINC00261, LINC00312, MEG3, PVT1, SUMO1P3, TERC.
- **Glioma:** H19, HOTAIR, MEG3.
- **Kidney Cancer:** AFAP1-AS1, DGCR5, HIF1A-AS2, WT1-AS.
- **Laryngeal Squamous Cell Carcinoma:** HOTAIR, MALAT1.
- **Leukemia:** DLEU2, GASS, H19, HOTAIR, HOXA-AS2, MALAT1, MEG3, TERC, TUG1, XIST.
- **Liver Cancer:** H19, HEIH, HOTAIR, HOTMIP, HULC, KCNQ1OT1, MALAT1, MEG3, MIR7-3HG, PANDAR.
- **Non-Small Cell Lung Cancer:** BANCR, CCAT2, GASS6-AS1, HOTAIR, MALAT1, MEG3.
- **Other Lung Cancers:** ACTA2-AS1, LUCA1.
- **Melanoma:** BANCR, HOTAIR, PTENP1.
- **Multiple Myeloma:** MEG3, PVT1.
- **Nasopharyngeal Carcinoma:** HOTAIR, LINC00312.
- **Neuroblastoma:** HAND2-AS1, SNHG16.
- **Oesophageal Squamous Cell Carcinoma:** LINC01234.
- **Osteosarcoma:** TUG1.
- **Ovarian Cancer:** H19, HOTAIR, LSINCT5, WT1-AS, XIST.
- **Pancreatic Cancer:** GASS, HOTAIR, PVT1.
- **Pituitary Cancer:** MEG3.
- **Prostate Cancer:** CBR3-AS1, GASS, HOTAIR, LINC00963, MALAT1, PCA3, PCAT1, PCGEM1, PRNC1R1, PVT1, TERC, XIST.
- **Rhabdomyosarcoma:** RMST.
- **Sporadic Pediatric Adrenocortical Tumors:** KCNQ1OT1.
- **Testicular Cancer:** XIST.
- **Thyroid Cancer:** NAMA, PTSC3.

Oncogenes:
- ACTA2-AS1, AFAP1-AS1, BANCR, BCAR4, BLACAT1, CBR3-AS1, CCAT1, CCAT2, CRNDE, H19, HAND2-AS1, HEIH, HIF1A-AS2, HNF1A-AS1, HOTAIR, HOTMIP, HOXA-AS2, HULC, JADRR, KCNQ1OT1, LINC00152, LINC00963, LSINCT5, LUCA1, MALAT1, MIR155HG, MIR17HG, PCA3, PCAT1, PCGEM1, POU5F1P5, PRNC1R1, PVT1, SNHG16, SPRY4-IT1, SUMO1P3, TERC, TRERNA1, TUG1, UCA1, XIST.

Tumor Suppressor Genes:
- ADAMTS9-AS2, CAHM, DLEU2, DLX6-AS1, GACAT1, GASS, GASS6-AS1, GNAS-AS1, LINC00261, LINC00312, MEG3, MIR31HG, MIR7-3HG, NAMA, NEAT1, PTSC3, PTSC3, PTENP1, TERC, TUSC7, WT1-AS, ZFAS1.

Other Cancer-Related IncRNAs:
- AIRN, EMX2OS, FTX, HIF1A-AS1, HOTAIRM1, HOXA11-AS, IPW, KRASP1, LINC00538, LINC00887, LINC01233, NBR2, NRON, RMRP, RPS6KA2-AS1, TSIX.
Serum sample of NSCLC (200ul each, n=3)
Serum samples of healthy donors (200ul each, n=24)
Geomean of 3 ref used for normalization.
Genomic contamination control was used to assure no genomic DNA contamination that may affect result (Cq>30).
Forty-one cancer-related IncRNAs showed detectable signal (Average Cq<30) in NSCLC patient serum samples
The PVT1 and RMRP showed significant up-regulation (77-fold and 24-fold, p<0.01, Cq<25 in cancer samples).
Serum sample of NSCLC (200ul each, n=3)
Serum samples of healthy donors (200ul each, n=24)
Geomean of 3 ref used for normalization.
Genomic contamination control was used to assure no genomic DNA contamination that may affect result (Cq>30).
Forty-one cancer-related lncRNAs showed detectable signal (Average Cq<30) in NSCLC patient serum samples
The PVT1 and RMRP showed significant up-regulation (77-fold and 24-fold, p<0.01, Cq<25 in cancer samples).

What role may PVT1 be playing?
(A) In normal cells, MYC is targeted by phosphorylation on threonine 58 (Thr58) and becomes destabilized and degraded. (B) In cancer cells, gain of 8q24 promotes the expression of MYC and PVT1. PVT1 interferes with the phosphorylation of Thr58 on MYC, which stabilizes the protein and increases its level.
Long non-coding RNA (IncRNA)

- Introduction to long-noncoding RNA
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- 3 step process to microRNA biomarker discovery
  - Application: microRNAs from blood for liver toxicity

- Summary
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.
Unique miRNA signatures are found in human cancer

- miRNAs located in genomic regions amplified in cancers (e.g. miR-12-92 cluster) can function as oncogenes, whereas miRNAs located in portions of chromosomes deleted in cancers (e.g. miR-15a-miR-16-1 cluster) can function as tumor suppressors.

- Abnormal expression of miRNAs has been found in both solid and hematopoietic tumors.

- miRNA expression fingerprints correlate with clinical and biological characteristics of tumors, including tissue type, differentiation, aggression and response to therapy.

- The race is on to develop miRNA biomarkers and therapeutics!
## Circulating miRNA biomarkers in the press

### No shortage of candidate genes

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Deregulation in cancer</th>
<th>Theragnostic and prognostic value</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>AUC</th>
<th>P value</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>linc-7a</td>
<td>Decrease in gastric cancer</td>
<td>Discriminate gastric cancer from healthy controls</td>
<td>--</td>
<td>--</td>
<td>0.002</td>
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<td>Tsujura et al. (2010)</td>
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<td>linc-7f</td>
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<td>Associated with overall survival in NSCLC</td>
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<td>0.036</td>
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<td>Silva et al. (2011)</td>
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<td>miR-1</td>
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<td>--</td>
<td>--</td>
<td>&lt;0.001</td>
<td>--</td>
<td>Hu et al. (2010)</td>
</tr>
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<td>--</td>
<td>0.014</td>
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<td>Roth et al. (2010)</td>
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<td>miR-17</td>
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<td>52%</td>
<td>93%</td>
<td>0.743</td>
<td>0.0001</td>
<td>Zhou et al. (2010)</td>
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<td>miR-17 + 10a</td>
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<td>Discriminate gastric cancer from healthy controls</td>
<td>63%</td>
<td>80%</td>
<td>0.741</td>
<td>0.0002</td>
<td>Zhou et al. (2010)</td>
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<td>64%</td>
<td>70%</td>
<td>0.717</td>
<td>&lt;0.0001</td>
<td>Ng et al. (2009)</td>
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<td>miR-17-5p</td>
<td>Increase in gastric cancer</td>
<td>Discriminate gastric cancer from healthy controls</td>
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<td>Tsujura et al. (2010)</td>
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<tr>
<td>miR-20b</td>
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<td>Associated with advanced stages and lymph node metastases in NSCLC</td>
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<td>&lt;0.01</td>
<td>--</td>
<td>Silva et al. (2011)</td>
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<td>Rossi et al. (2010)</td>
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<td>miR-21 + 126 + 210 + 486-5p</td>
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<td>Discriminate gastric cancer from healthy controls</td>
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<td>0.006</td>
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<td>Discriminate pancreatic adenocarcinoma from healthy controls</td>
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<td>89%</td>
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<td>miR-23a</td>
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<td>83%</td>
<td>85%</td>
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<td>Huang et al. (2010)</td>
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<td>miR-29b</td>
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<td>Associated with progression in CLL</td>
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<td>&lt;0.001</td>
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<td>Viscione et al. (2009)</td>
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<td>Hu et al. (2010)</td>
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<td>0.01</td>
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<td>Roth et al. (2010)</td>
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<td>Increase in CRC</td>
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<td>70%</td>
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<td>miR-92a</td>
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<td>Discriminate CRC from healthy controls</td>
<td>84%</td>
<td>71%</td>
<td>0.838</td>
<td>&lt;0.0001</td>
<td>Huang et al. (2010)</td>
</tr>
</tbody>
</table>

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**Cho, (2011) Front. Gene. 2.**
Stable extracellular miRNA in blood

- High levels of nucleases present in plasma
  - Freely circulating RNA *should be rapidly degraded*
  - Surprisingly, stable miRNA can be detected in serum and plasma

- But does it matter whether it comes from exosomes or Ago2 or…?
  - That depends
Exosomes: small membrane vesicles (30–100 nm), secreted by most cell types into the bloodstream.

**Functional biomolecules:**
- DNA fragments (exosomal DNA, exoDNA)
- Proteins and/or peptides
- mRNA
- microRNA (miRNA)
- Lipids

- Exosomes play a central role in cell-to-cell communication.
- The majority of DNA associated with tumor exosomes is double-stranded, representing whole genomic DNA.
- Biological molecules (protein, RNA, and miRNA) contained in exosomes are well protected by a lipid bilayer membrane that confers a high degree of stability.

Challenges:

- Many current protocols to isolate vesicles use ultracentrifugation
- Long processing time, and the process is unrepeatable and not selective for tumor exosomes

Current methods

- Differential centrifugation
- Size exclusion
- Immunoaffinity isolation
- Microfluidic devices
- Polymeric precipitation (ExoQuick)

Issues

- Long processing time
- Cannot achieve absolute separation
- Lower yields
- Low purity
- High contamination

Needs

- Rapid, exosome-specific extraction
- Simple, effective release from exosomes
- Highly sensitive, specific detection of endogenous biomolecules

Case study: acetaminophen poisoning in humans

‘Tylenol’ overdose is one of the most common causes of poisoning worldwide. In the US and UK it is the most common cause of acute liver failure.

0-24 hours mild symptoms
24-48 hours symptoms of acute liver poisoning
3 to 5 days complete hepatic failure, kidney failure, cerebral edema, sepsis, multiple organ failure, and death

Can we find miRNA markers of Tylenol poisoning and can we distinguish this poisoning from other liver syndromes

With Dr. James Dear, Edinburgh University
Circulating miRNA as Biomarker

Phase I (determine expressed miRNAs):
Pooled sample profiling or NGS
Screen miRNome

Phase II (determine differentially expressed miRNAs):
Individual profiling of samples (that went into pools), n~40
Screen only expressed miRNAs (< 384-well plate)

Phase III (statistical power):
Individual profiling of all samples in study,
Using classifier candidate miRNAs (1-12-?)
miScript MirBase Profiler for version miRnome 21

- **Human**
  - Coverage through miRBase v21
  - 2402 qPCR assays
Phase 1: Determine expressed miRNAs

Survey the miRNA landscape for your system

- **54 plasma samples:** 27 control samples, 27 liver injury samples

- **Total RNA Isolation:** miRNeasy Serum/Plasma Kit
  - **Note:** Perform QC at this step to ensure samples are devoid of inhibitors

- **Prepare random pools, 10 samples per pool:** 2 control pools, 2 patient pools

- **miRNA Expression Profiling:** miScript PCR System and Human miRNome

**Which miRNAs were selected?**

- miRNAs expressed in all four pools
- miRNAs expressed in three pools
- miRNAs expressed in two pools
- miRNAs expressed in one pool

**~377 assays**
Phase 2: Determine differentially expressed miRNAs

Secondary screen of individual samples from Phase 1

- **54 plasma samples**: 27 control samples, 27 liver injury samples
- **miRNA Expression Profiling**: miScript PCR System and expressed miRNAs (377 assays)
  - replicate PCR reactions, 4 fluidigm biomark runs. 40K data points, 4 days

**Which miRNAs were differentially expressed?**

**Scatter Plot**

- miR-122-5p
- miR-885-5p

**Volcano Plot**

- miR-885-5p
- miR-122-5p
Phase 3: Statistical Power

**Develop training set of data on candidate markers**

- **Samples**: Screen larger cohort of affected individuals
- **Assays**: Re-array differentially expressed miRNAs
  - Include invariant miRNAs (identified by NormFinder, etc.) for data normalization
- **Continually refine classifier model**
  - Original 54 samples, top 16 miRNAs
Validate miRNA signature on naïve samples

Some things to consider at this point
- strong changes might be general indicators or even non-specific (e.g. other liver disease? Stress markers?)
- Relatively weak changes might add specificity
- High level of false positives is really undesirable

What’s next?
- Revisit literature, Ingenuity IPA, etc
- Screen other types of samples to help define specificity and refine signature.
Long non-coding RNA (IncRNA)

- Introduction to long-noncoding RNA
  - What is IncRNA?

- IncRNA and liquid biopsy
  - How IncRNAs can be part of a liquid biopsy program

- Integrating QIAGEN RT² IncRNA PCR Arrays into research
  - Application: IncRNA from serum & plasma

- Role of IncRNA
  - Function of IncRNA
  - Role of IncRNA in microRNA regulation

- Summary
  - Key messages

- Questions
Summary

- IncRNAs and miRNAs are circulating biomarkers ideal for “liquid biopsies”

- For projects involving miRNA and IncRNA we recommend the miRNeasy product line for total RNA isolation

- RT² IncRNA qPCR Arrays with Pre-Amplification can be used to identify circulating IncRNA biomarkers from biofluids.

- miScript miRbase Profiler contains 2402 miRNA assays for miRNome profiling which can be used with a 3 phase strategy for quick biomarker discovery
Questions?

Thank you for attending today’s webinar!

Samuel J. Rulli, Ph.D.

Contact QIAGEN
1-800-426-8157
BRCsupport@QIAGEN.com
QIAwebinars@QIAGEN.com