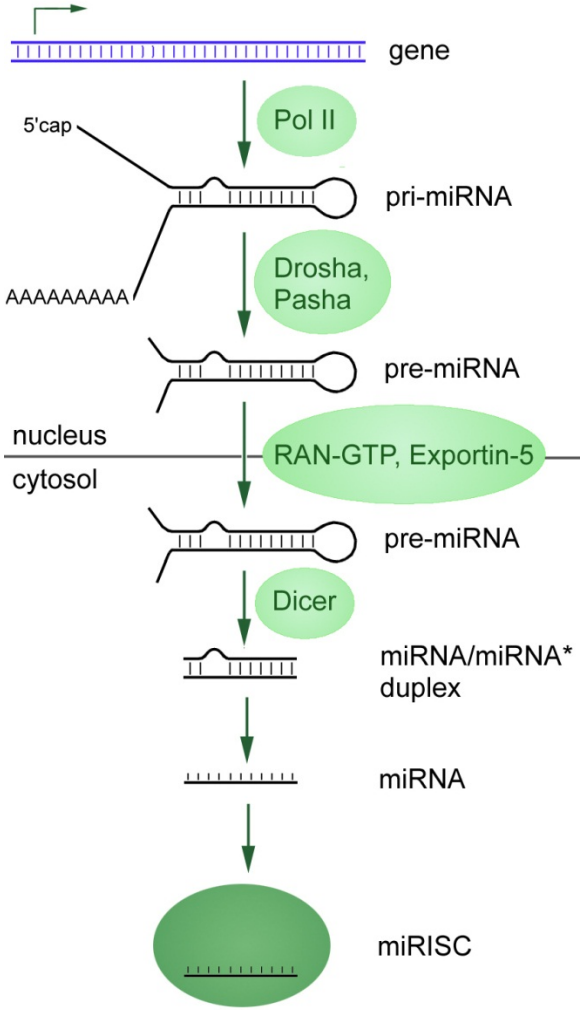
Assaying microRNAs in biofluids for detection of drug-induced cardiac injury

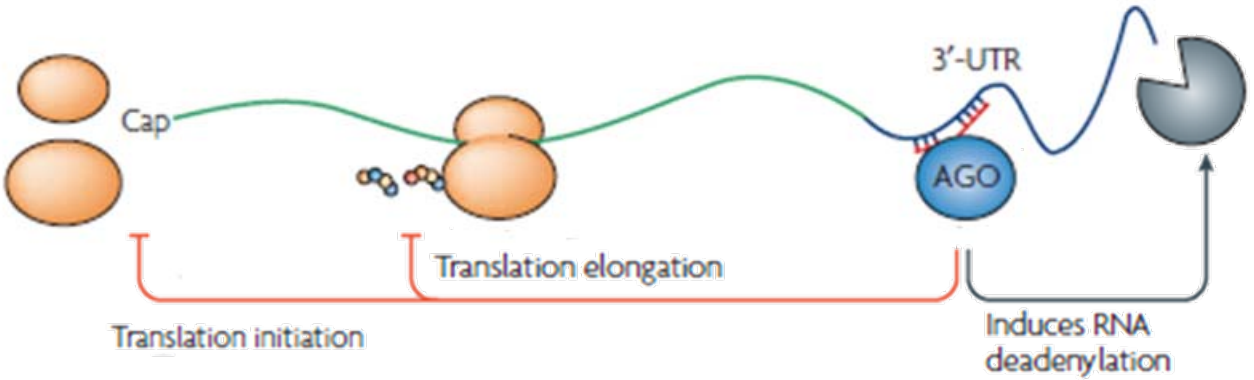
HESI Annual Meeting
State-of-the-Science Session
June 8, 2011

Karol Thompson, PhD
Center for Drug Evaluation & Research
US Food and Drug Administration
Silver Spring, Maryland

MicroRNAs are short, non-protein coding RNAs that are synthesized from intergenic regions and processed to a mature size of 21-24 nucleotides



Mature microRNAs repress gene expression by binding to homologous sequence in the 3' region of mRNAs as part of a ribonucleoprotein complex.



From Hutvagner and Simard. Argonaute proteins; key players in RNA silencing. Nature Reviews Molecular Cell Biology 9:22,2008.

HESI technical committee on the application of genomics to risk assessment

- Formed in 1999 to develop a collaborative scientific program to address issues, challenges, and opportunities afforded by the emerging field of toxicogenomics.
- MicroRNA was adopted as a new project area at the October 2009 plenary meeting
  - Potential utility in toxicology remains a gap
- MicroRNA working group
  - Co-Chairs: Tim Schaiff (Pfizer), Philippe Couttet (Novartis), and Karol Thompson (CDER, FDA)
  - Raegan O'Lone (HESI program manager)
- Workshop held on October 19, 2010 to assess the state-of-the-science on use of microRNAs in toxicological applications

Workshop Agenda

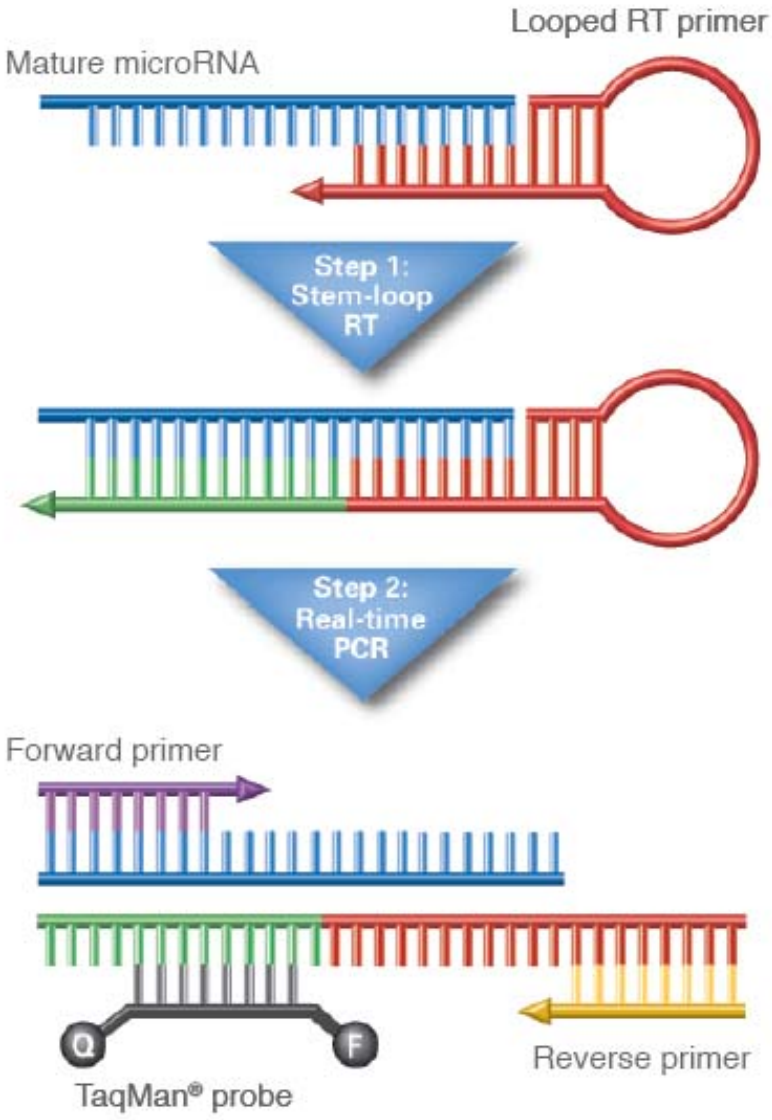
- **Progress on the use of microRNAs as biomarkers of injury**
  - Evaluation of techniques for genome-wide miRNA measurements …… ……....Dr Graham Brock, Pfizer
  - Issues associated with microRNA measurements… Dr Kai Wang, ISB
  - MicroRNAs as injury markers in urine……...Dr Peter Yuen, NIDDK
  - MicroRNAs as injury markers in tissue..Dr Philippe Couttet, Novartis
- **Design of studies to assess microRNAs as injury markers**
  - Biomarkers of cardiotoxicity……....Dr Greg Falls, GSK
  - Biomarkers of nephrotoxicity…… ……Dr Jean-Charles Gautier, sanofi-aventis
  - miR-122 as a hepatotoxicity biomarker…..Dr Ameesha Batheja, J&J
  - Biomarkers of testicular toxicity……..Dr Hungyun Lin, Pfizer

Potential utility of circulating microRNAs as injury biomarkers

- Small total number of microRNAs compared to mRNAs (~1000 human miRNAs)
- A few microRNAs exhibit highly specific tissue expression
- microRNAs are rapidly released from tissues into circulation with development of pathology
  - Tissue-selective microRNAs may be useful circulating biomarkers of tissue injury at specific sites
- Extracellular microRNAs are stable in blood and urine
  - *However, purified microRNAs are rapidly degraded when added to blood*
- Low barrier for new assay development
  - *High conservation of sequence across species*
- microRNA can be quantitated using highly sensitive, modified RT-qPCR methods
  - *Most methods extend the length of microRNAs prior to PCR*

- One approach* uses a target-specific stem-loop RT primer (specific to the mature miRNA target) to extend the length of the microRNA at the 3’ end of the microRNA.

*Applied Biosystems Taqman microRNA assays



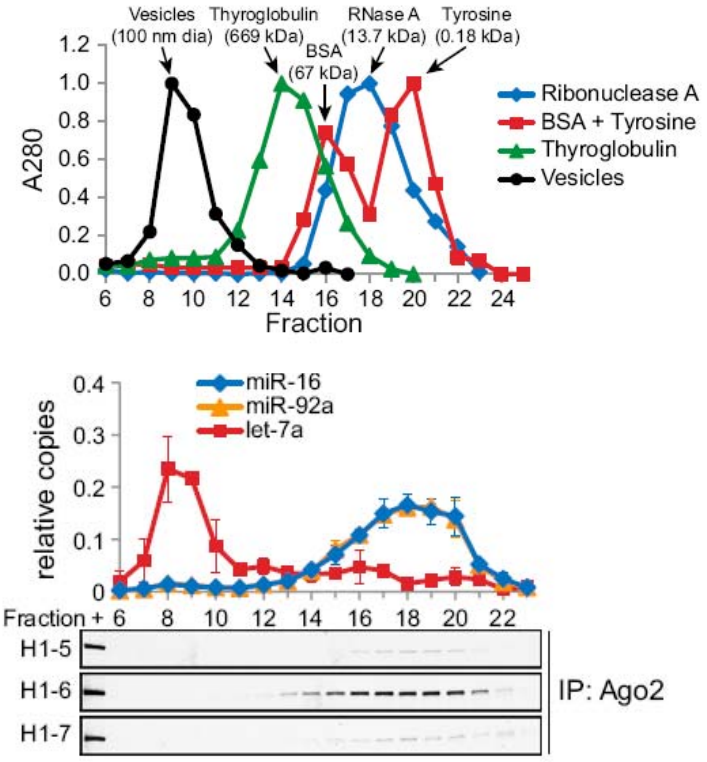
Workshop Summary: Challenges with the use of circulating microRNAs as biomarkers of injury

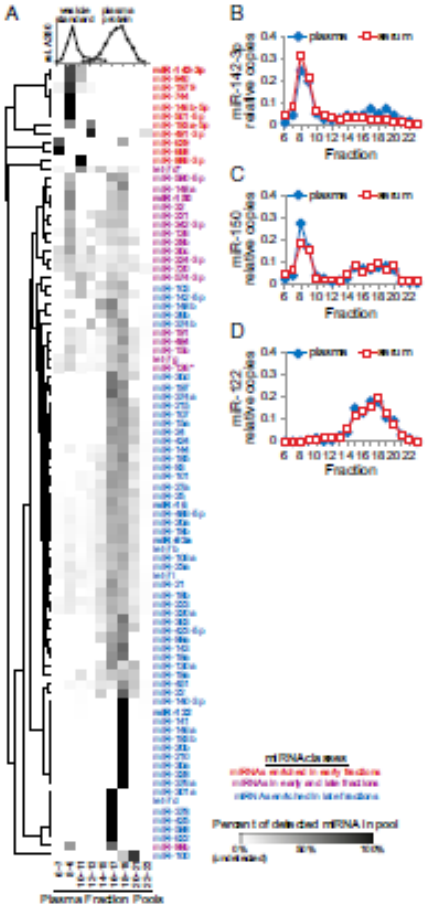
- Short sequence length is a challenge to hybridization specificity
  - Poor comparability between microRNA microarray platforms
- Heterogeneity in microRNA length - “isomirs”
- Potential assay interference from precursor forms of microRNA
- Biofluids contain inhibitors of RT-qPCR enzymes
- MicroRNAs in blood cells can contaminate biofluid samples

Workshop Summary: Challenges with the use of circulating microRNAs as biomarkers of injury

- microRNA levels in biofluids too low to quantitate
- Preamplification PCR steps required to measure in biofluids - introduces bias?
- No consensus on endogenous microRNA controls in biofluids
- Quantitative or qualitative difference in microRNAs recovered from serum and plasma? Effect of serum/plasma protocol?
- Non-homogenous physical state of microRNAs in circulation - associated with protein and/or encapsulated in lipid vesicles in plasma/serum

- **The majority of circulating microRNAs in human plasma are found in protein complexes that contain Ago2**





From Arroyo et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. PNAS 2011 (prepublication)

Collaborative study on the use of microRNAs in toxicological applications

- Identify key pre-analytical variables for the successful quantitation of injury-related microRNAs in serum, plasma, and urine. Verify methods that can be implemented in pre-clinical toxicology studies.
- In a model of drug-induced tissue injury, assay microRNAs in biological samples, anchored to protein biomarkers and histopathology
- Samples will be generated from an in-life study run at a central site
- Samples will be distributed to multiple laboratories and analyzed using a **standard protocol** and defined protocol modifications. All protocols involve absolute quantitation of microRNA levels.

Model system: Isoproterenol-induced myocardial injury in rats

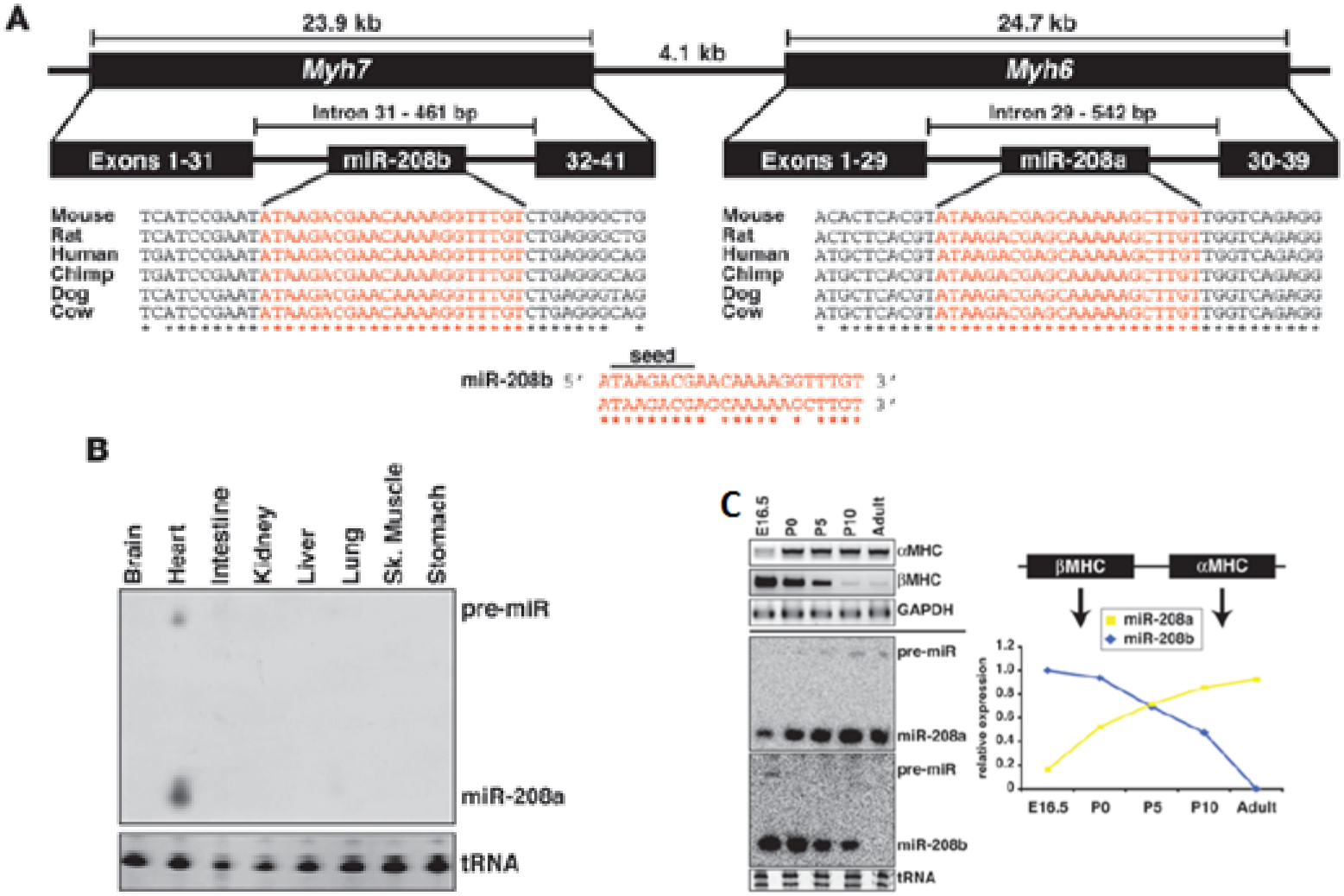
- Phase I: Dose selection study
  - **Interim endpoint**: Level of serum cardiac troponin I (cTnI) 4 hr after single sc dose of 0, 0.5, 1, 2, or 4 mg/kg isoproterenol in male Hanover Wistar rats
    - Doses based on study from HESI Troponins WG
  - **Terminal endpoint**: Histopathology of the heart at 24 hr after single sc dose of 0, 0.5, 1, 2, or 4 mg/kg isoproterenol in male Hanover Wistar rats to confirm necrosis.
- Phase II: Serum *vs* Plasma comparison in an injury model

Evidence of miRNAs as circulating markers of acute drug-induced cardiac injury

- Plasma miR-208 as a biomarker of isoproterenol induced myocardial injury in the rat
  - Ji *et al.* Clin Chem 55: 11, 2009
  - Plasma miR-208a elevated 3-24 h after dosing
  - Similar time course to cTnI
- Circulating microRNAs associated with AMI
  - miR-208 [2, 4] (Cardiac muscle specific)
  - miR-499 [1, 2, 3, 4] (Cardiac muscle enriched)
  - miR-1 [3, 4, 5] (Enriched in muscle - not selective for cardiac)
  - miR-133a/b [3, 4] (Enriched in muscle - not selective for cardiac)

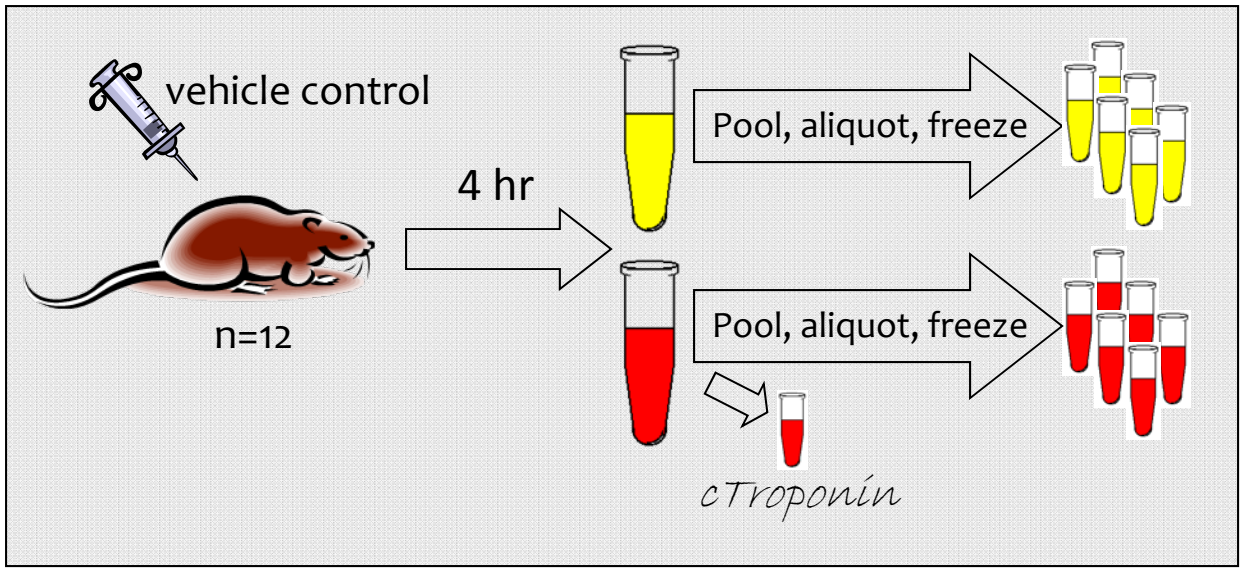
(1) Adachi et al. Clin Chem 56:7, 2010; (2) Corsten et al. Circ CV Genet 3:499, 2010; (3) D'Alessandra et al. Eur Heart J 31:2765, 2010; (4) Wang et al. Eur Heart J 31:659, 2010; (5) Ai et al. BBRC 391:73, 2010

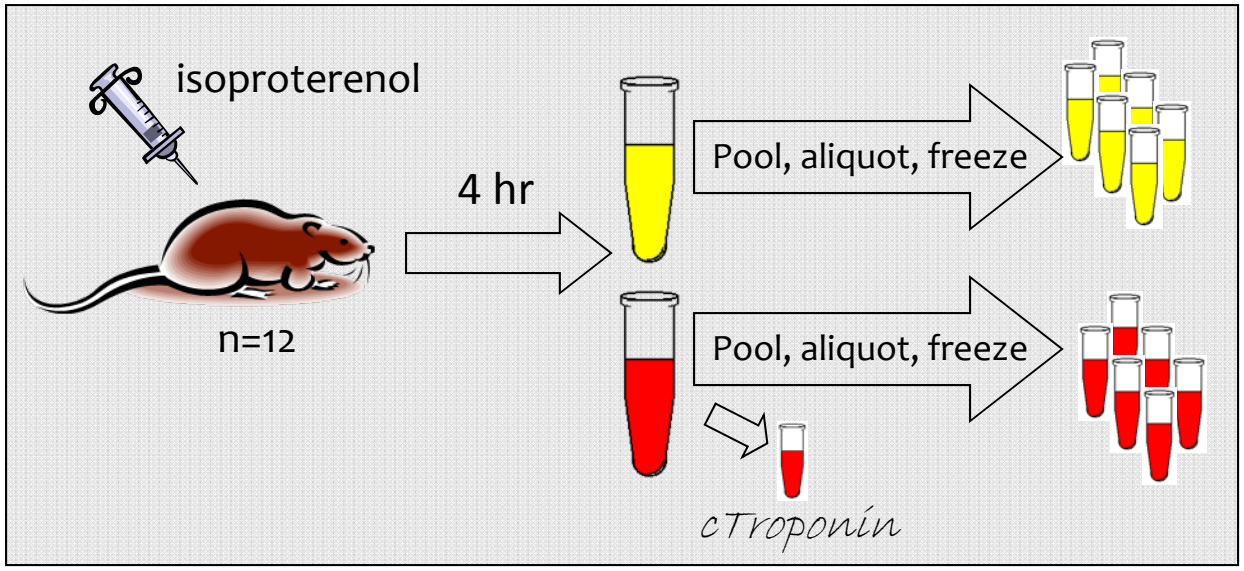
Cardiac-specific expression of miR-208a and miR-208b parallels their host genes Myh6 and Myh7

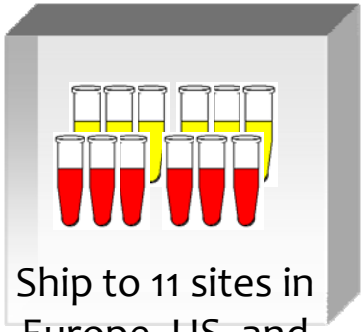


From Callis *et al.* J Clin Invest 119:2772-86, 2009

Sample generation for Phase II





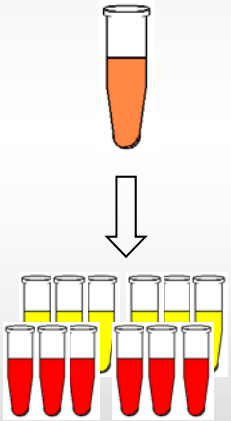


Ship to 11 sites in Europe, US, and Japan

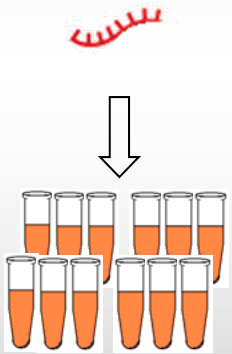
Standard protocol for microRNA analysis in Phase II

Step 1 - MicroRNA Isolation - Qiagen miRNeasy mini kit

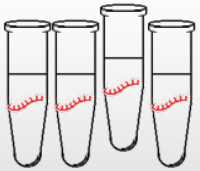
Lysis Buffer



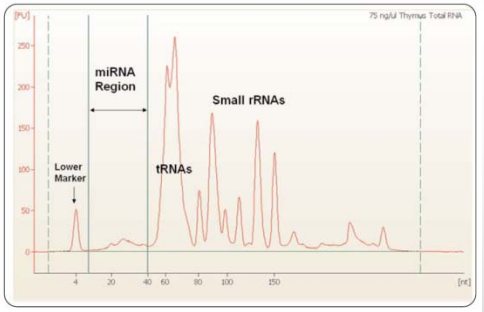
Ath-159a



Spin column purification

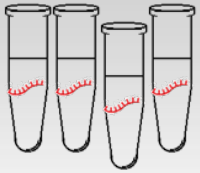


Qualitative check on Agilent Pico RNA chip



Standard protocol for microRNA analysis in Phase II

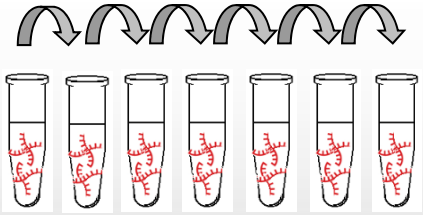
Steps 2 & 3 - Multiplexed Reverse Transcription and Preamplification



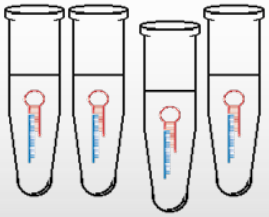
MicroRNA preps from serum and plasma

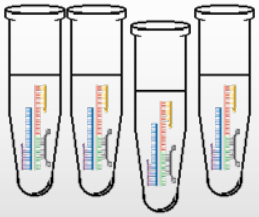
Reverse transcription using Megaplex RT primers (Rodent Pool A v2.0 - stem loop RT primers for 226 rat miRNAs + ath-159a)

Preamplification using Megaplex preAMP primers (for 226 rat miRNAs + ath-159a)



Serial dilutions of synthetic targets (miR-1, miR-208a, miR-499, miR-16)



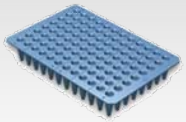


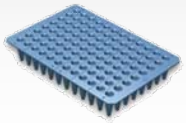
12 cycles

Standard protocol for microRNA analysis in Phase II

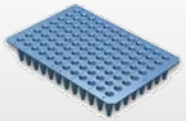
Step 4 - Real Time PCR Amplification

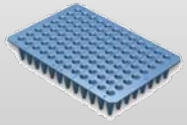
Taqman microRNA assays


miR-208


miR-1


miR-499

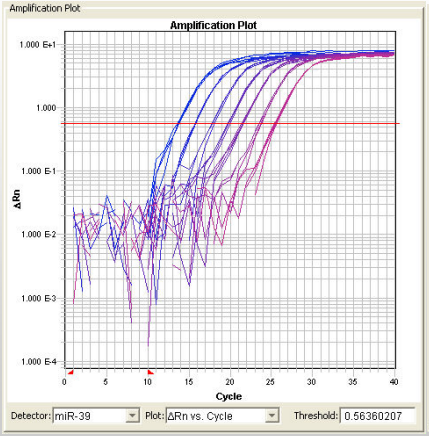

miR-16

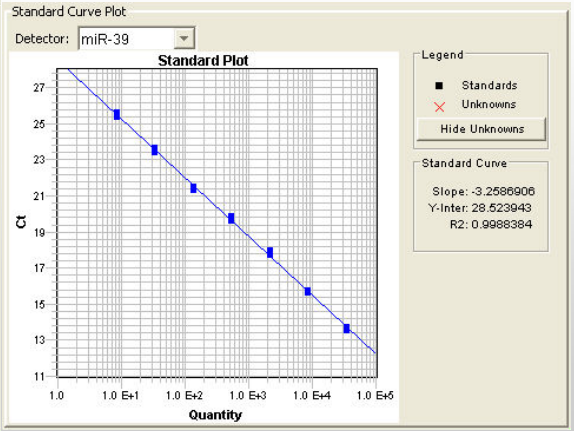

ath-miR159

Normalize using Ath-miR159 Ct

Absolute quantitation of target by calculating copy number from standard curve

Report microRNA levels as copy number per mL serum or plasma





Analysis of results from Phase II

- Variables include
  - Site to site (~10 sites)
  - Reproducibility between technical replicates
  - Serum vs plasma
  - Treatment - vehicle control vs isoproterenol
  - Standard protocol vs variations
  - microRNA targets (for cardiac injury)
    - Limits of detection for each target from standard curves
  - Normalization methods
  - Protein-associated vs lipid vesicle-associated targets (optional targets)

MicroRNA Collaborative Study Phase III - Urine *vs* Blood sample in injury model

- Can injury-associated microRNAs detected in plasma also be detected in urine?
- What is the optimal protocol for detection of injury-associated microRNAs in urine?
- In-life study design: same model system as Phase II?
- Overnight urine collection at 18-24 hr time point
- Urine sampling: neat vs exosome enrichment? Exosome enrichment protocol?
- Reference miRNA for urine TBD

Anticipated Results

- Better understanding of preanalytical steps affecting microRNA detection and quantification in biofluids in drug-induced injury models
- Establish reference data set for comparing the sensitivity of methods for measuring injury-associated microRNAs in blood
- Important first steps in the exploration of the utility of circulating microRNAs as biomarkers of drug-induced injury beyond “proof of concept” studies

Thank you

- Abbott
- Actelion
- Allergan
- Amgen
- Astellas
- AstraZeneca
- Bayer Healthcare
- Biologie Servier
- GlaxoSmithKline
- Johnson & Johnson
- Eli Lilly
- Novartis Pharma AG
- Pfizer
- Sanofi-aventis
- Takeda
- Maastricht University, NL
- CDER, FDA
- NCTR, FDA
- NIDDK
- NIEHS
- NIST