Effects of Pre-analytic Variables on Circulating MicroRNAs Using a CCSG Biorepository

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What are MicroRNAs?

- Small RNA molecules (~21 nt)
- Found in almost every species
- Highly evolutionarily conserved
- Regulate gene expression

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Human MicroRNAs

- Currently there are 1,048 human microRNAs annotated in miRBase (Release 16.0, Dec. 2010)
- On average, each microRNA has 200 targets
- Over 60% of all human protein encoding genes are regulated by microRNAs
- Important functions in every aspect of the biology

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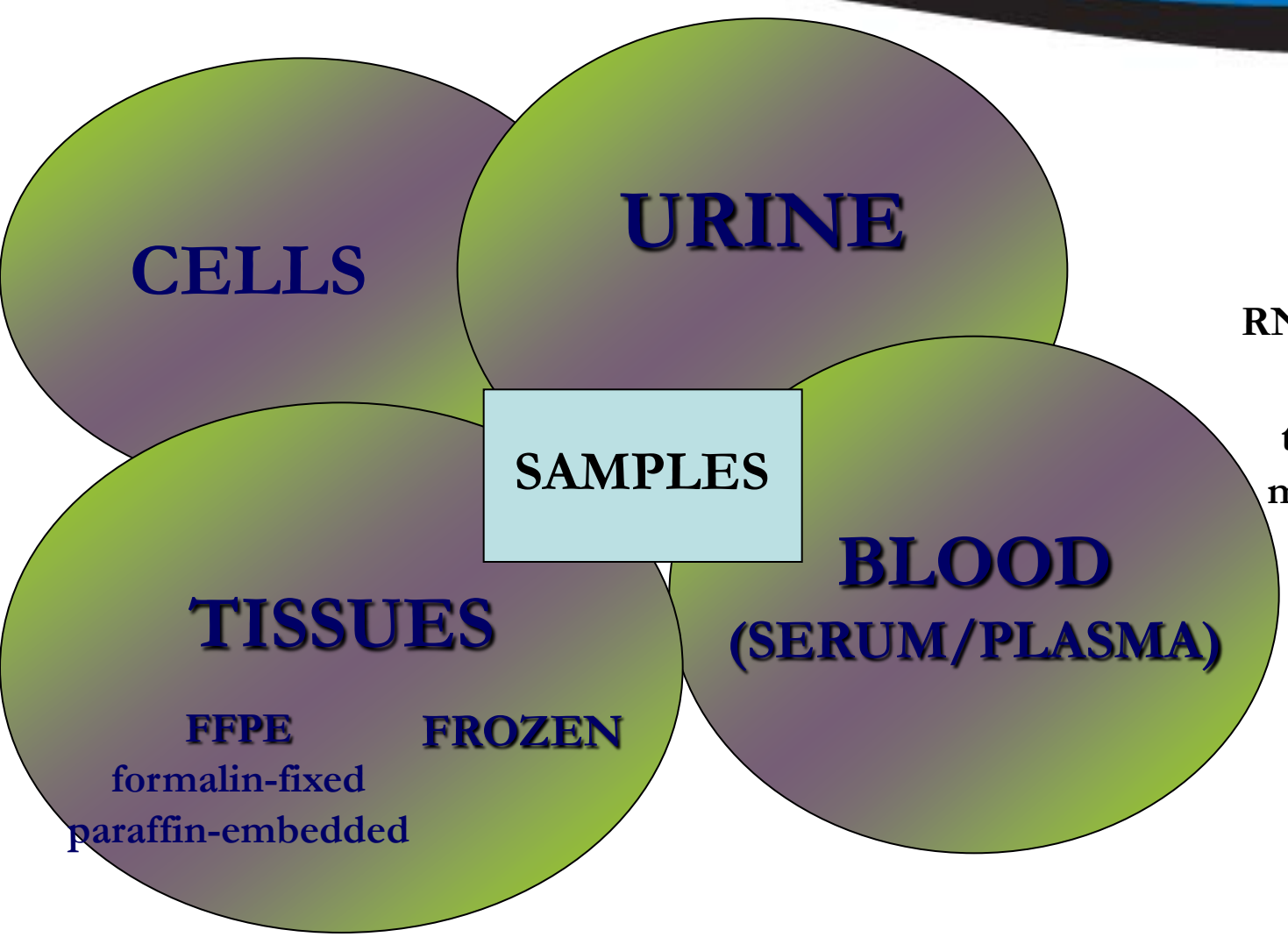
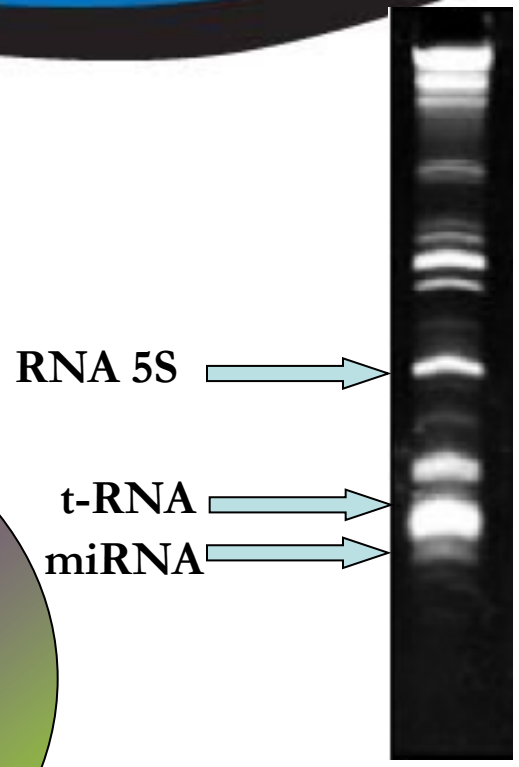
MicroRNAs and Cancer

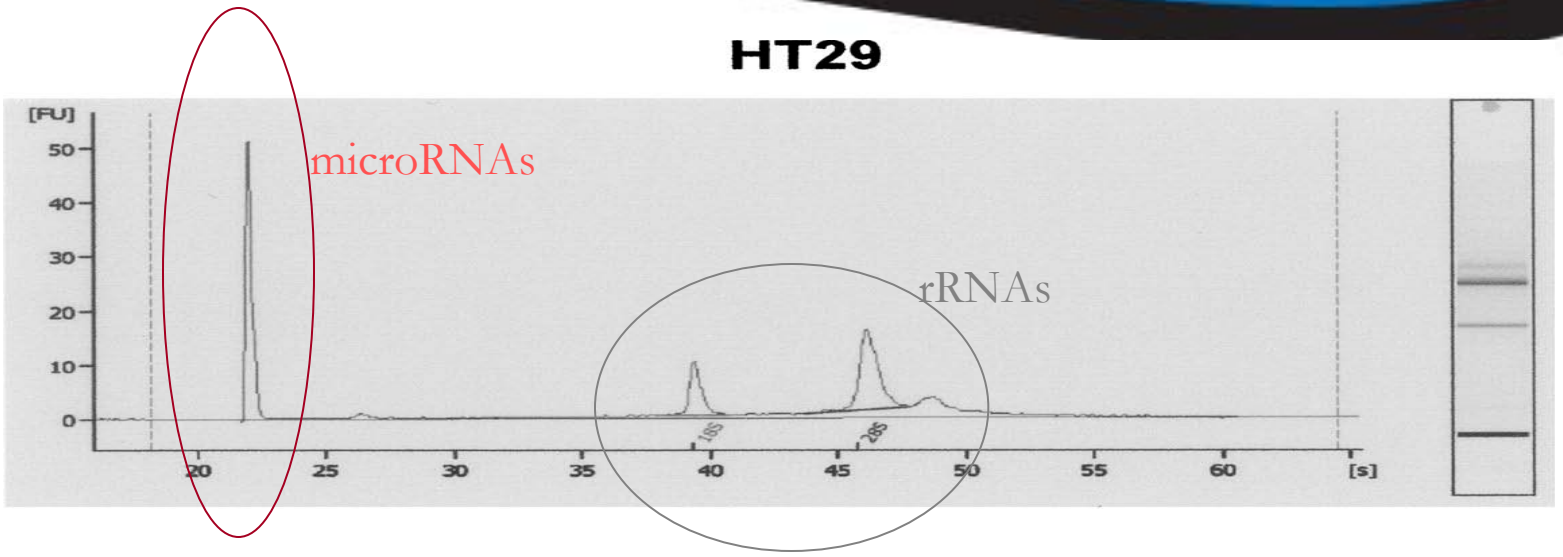
- microRNA may play a role as tumor suppressor genes (down regulated in cancer) or oncogenes (up regulated in cancer)
- microRNA disregulation is involved in initiation, progression, and resistance to therapy of human cancers
- microRNA as promising biomarkers of cancers

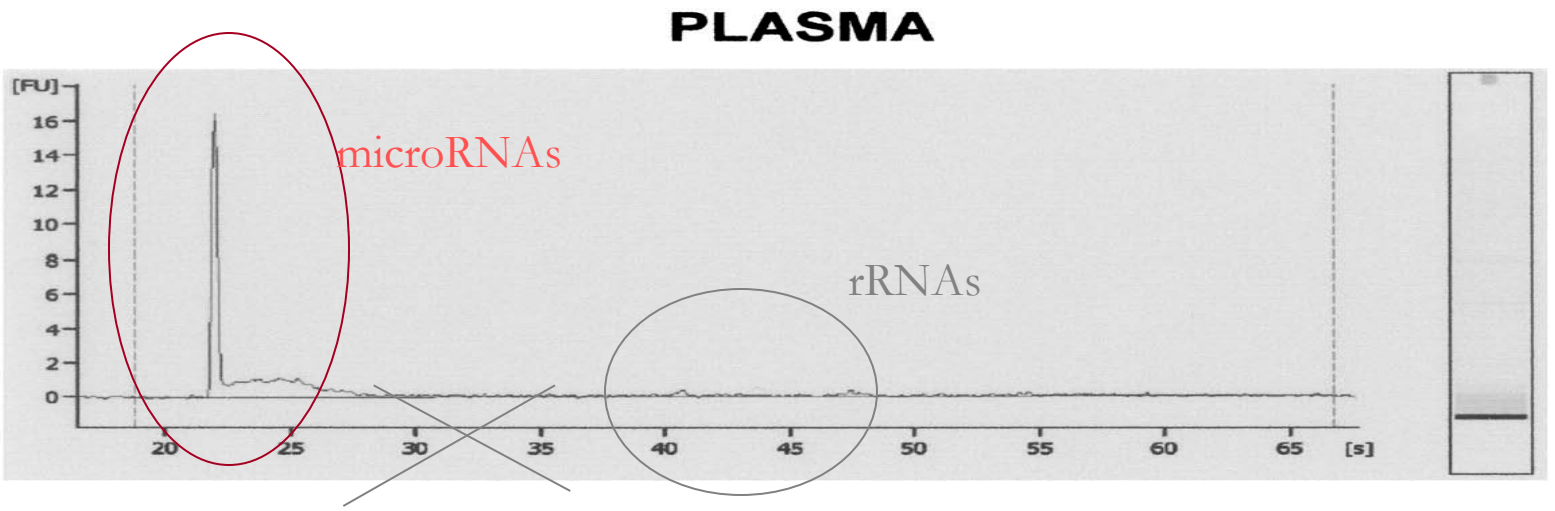
MicroRNAs Detected in:





Plasma RNAs from Colon Cancer Patients



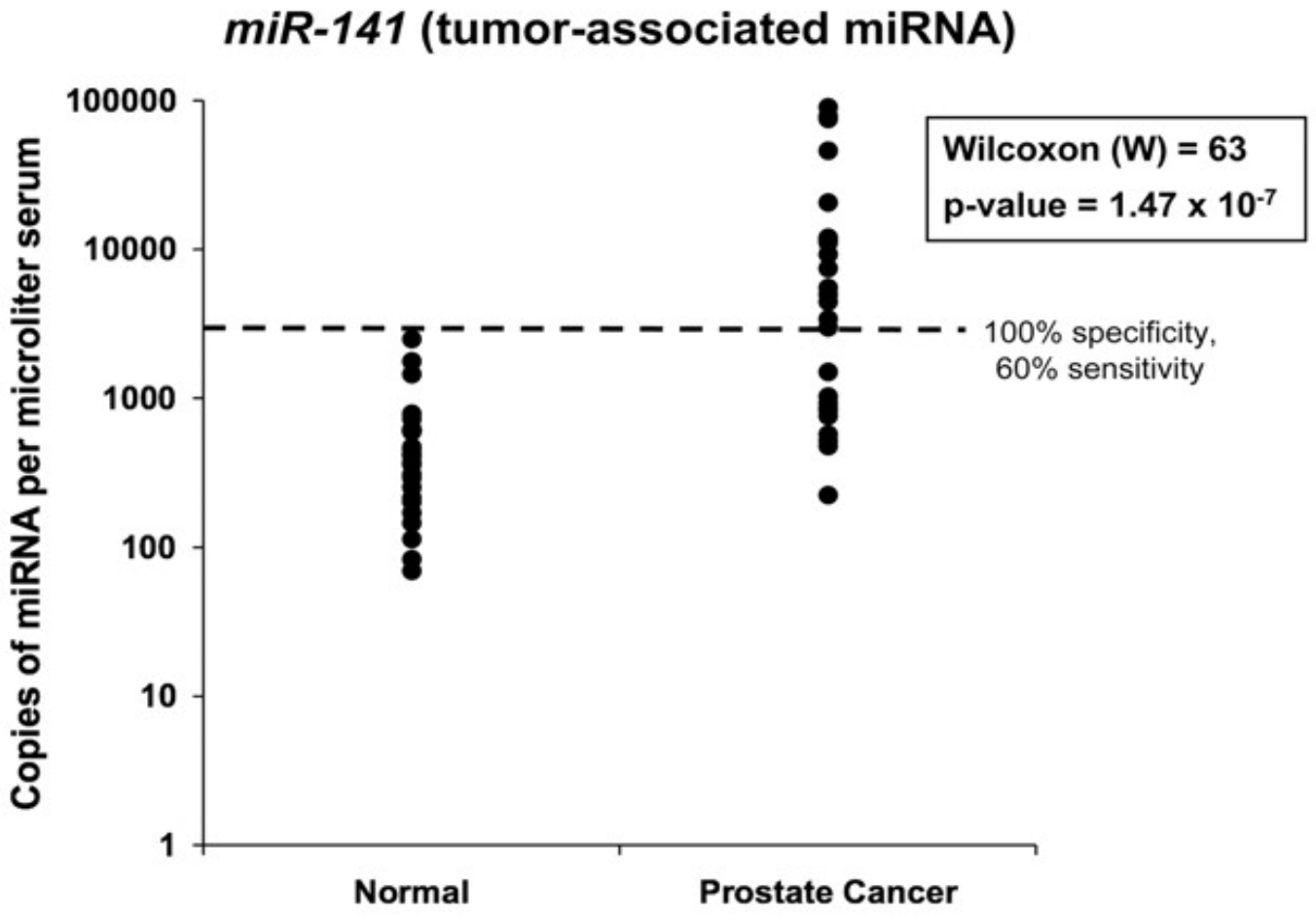


RNA 2008 14: 1424-1432

Circulating microRNAs and Human Cancer

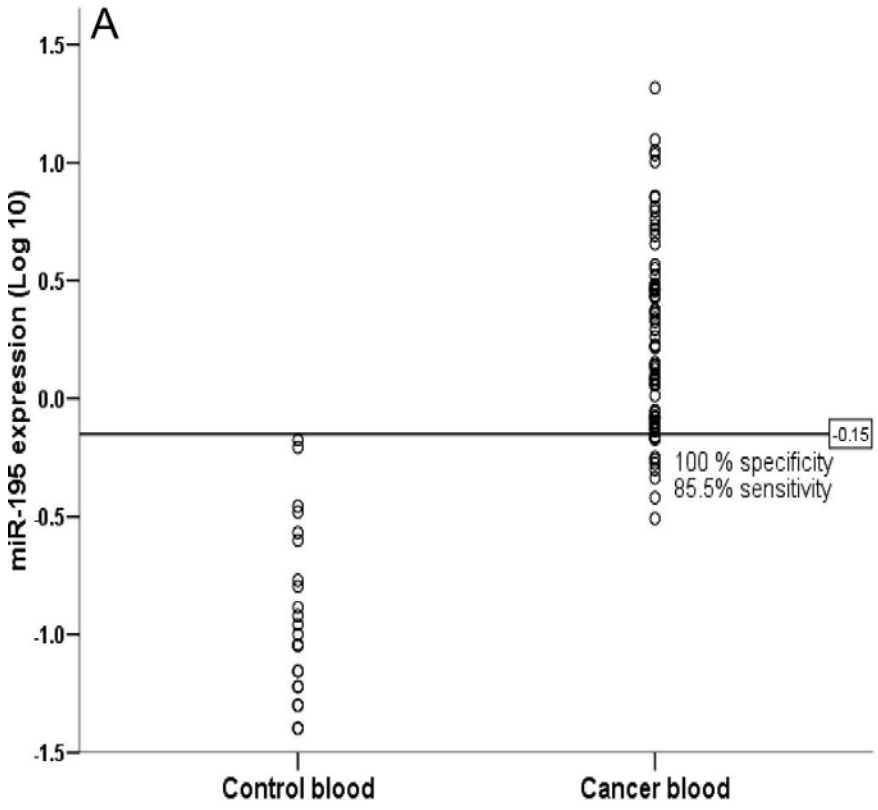
- Around 30 studies have been done in a variety of cancers, including colorectal, prostate, ovarian, breast, lymphoma, etc.
- Biospecimens include: serum, plasma, and whole blood.
- A few microRNA based biomarkers have been reported:
  - miR-141 in prostate cancer
  - miR-195 and let-7a in breast cancer
  - miR-155, miR-210 and miR-21 in lymphoma, etc

Detection of Human Prostate Cancer by Serum *miR-141*

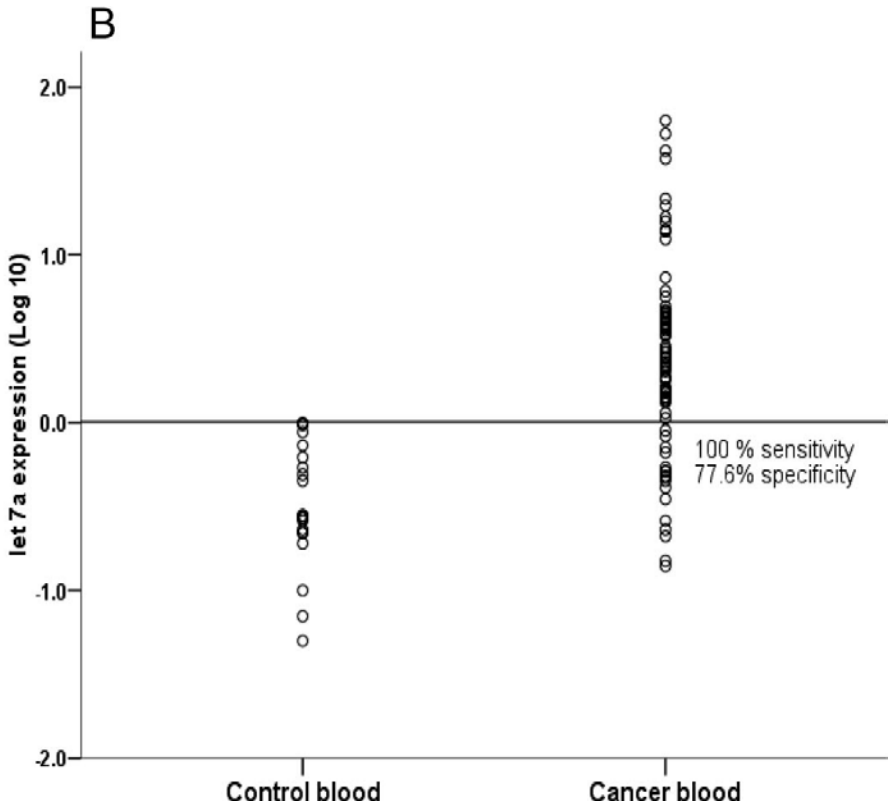


Mitchell P S et al. PNAS 2008

Blood *miR-195* and *let-7a* in Breast Cancer



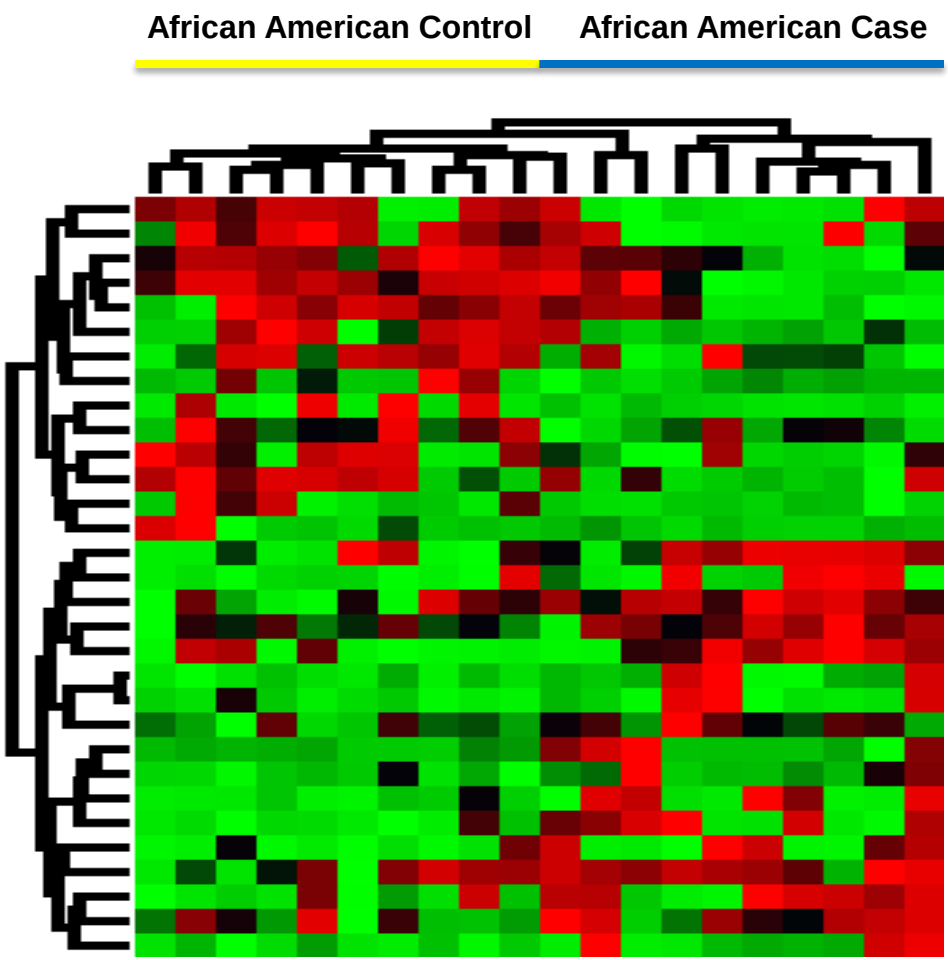
miR-195



Let-7a

Heneghan, et al, Annual of Surgery, 2010

Plasma microRNA Clusters in Breast Cancer Cases and Controls

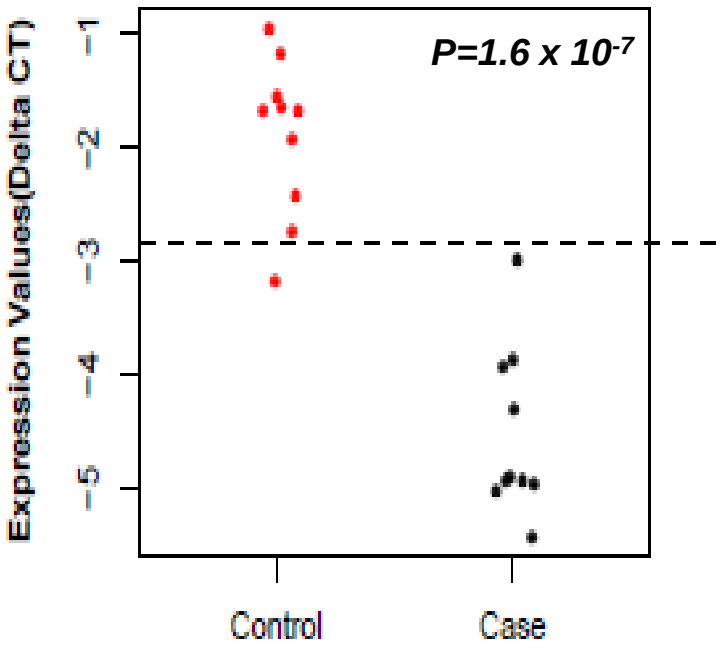


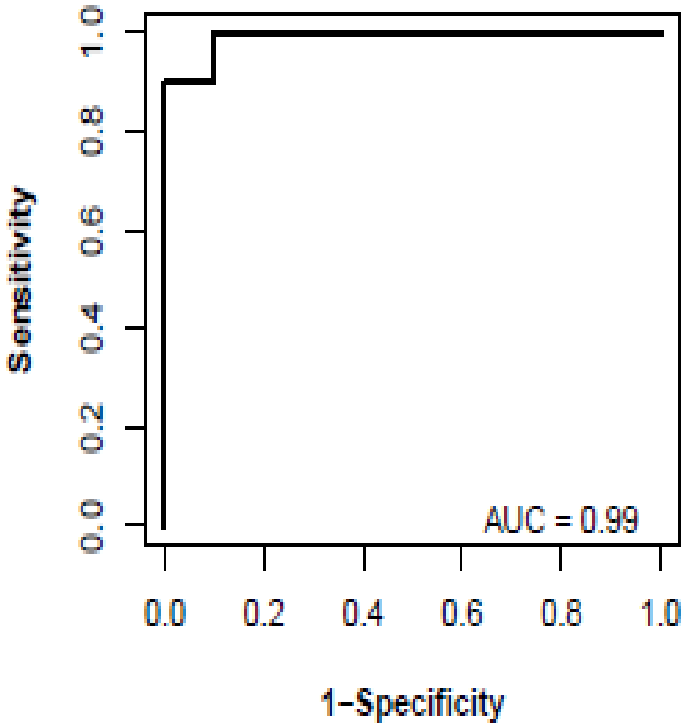
18 miRNAs were found to be differentially expressed between cases and controls, with 9 up-regulated in cases.

- Red: up-regulated / green: down-regulated

Zhao et al, PLOS ONE, 2010

Plasma Let-7d in Breast Cancer Cases and Controls





Challenges from Biospecimen Perspective

- No reliable internal control to normalize the circulating microRNA data
- Little is known about pre-analytic variables of circulating microRNAs
- No reliable QC tools available to assess the effects of pre-analytic variables on circulating microRNAs

Pilot study: Freeze/Thaw and Plasma MicroRNA

Study design

- Number of freeze/thaw: 1 vs 2
- Compare number of microRNAs that can be detected in plasma
- Compare expression of selected microRNAs used as internal controls in literatures (*miR-15* and *miR-16*)

Results

- 1 cycle: 137 microRNAs were detected
- 2 cycles: 117 microRNAs were detected
- The expression of *miR-15* was reduced 45% (1 cycle vs 2 cycles)
- The expression of *miR-16* was reduced 60% (1 cycle vs 2 cycles)

Conclusions

- The number of freeze/thaw affects circulating microRNA expression
- Biospecimen research in circulating microRNA is needed

Objectives of The Project

I. *Discover a panel of "housekeeping" circulating microRNAs which can be used as internal controls.*

II. *Development of circulating microRNA QC tools by studying the effects of pre-analytic variables on the internal control microRNAs identified in Objective 1.*

Scheme of Objective I

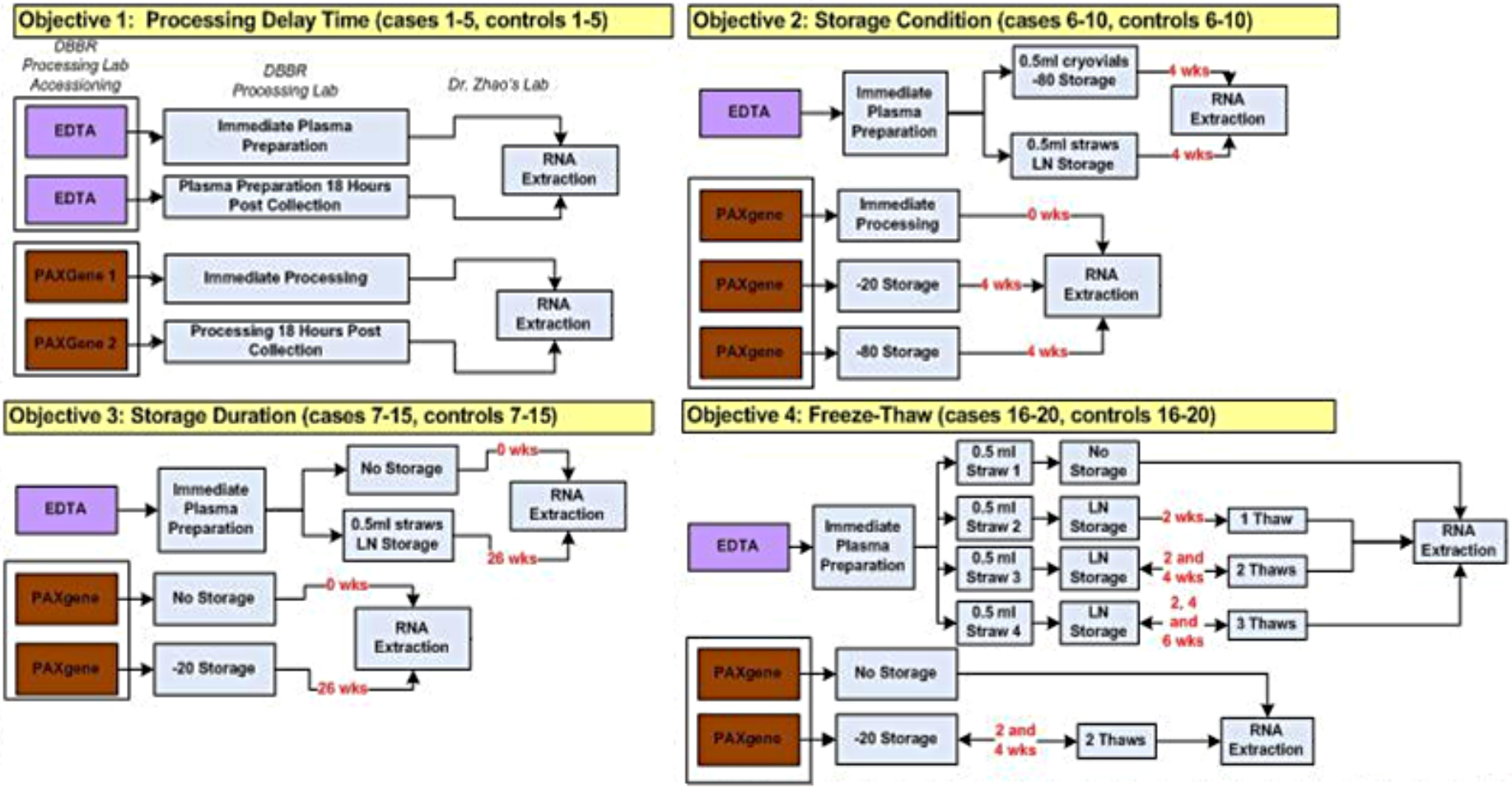
- Perform microRNA profiling in 40 plasma samples to identify a panel of circulating microRNAs based on the following criteria:
  - *occur in all tested samples*
  - *expression levels are not significantly different between cancer cases and controls*
  - *show little inter-individual variations among cases and controls*

- Further evaluated individually in an additional 200 plasma samples from 100 cancer cases and 100 healthy controls using quantitative real-time PCR based analysis

- In parallel, we will repeat microRNA profiling and validation analysis in the same study subjects as above, but using whole blood samples collected using PAXgene Blood RNA System

Scheme of Objective II

Analyzing the Effects of Pre-Analytical Variables on miRNAs



DataBank and BioRepository

- DataBank and BioRepository (DBBR) of Roswell Park Cancer Institute (RPCI) will be used to
  - *recruit study subjects*
  - *obtain high quality biospecimens*
  - *collect epidemiological/clinical data*
- Standard Operation Procedure (SOP) has been developed and strictly followed
- Blood collection, sample processing, and storage are completed in one hour

Overview of the Study Progress

- Study started in Nov. 2010
- So far, we have completed microRNA profiling in 40 study subjects (both plasma and whole blood)
- 152/200 (76%) of the study subjects needed for the validation analysis have been recruited

Cases and Controls in Microarray Profiling

20 Cases

- 8 prostate cancer patients (men) and 12 breast cancer patients (women)
- Average age of cancer diagnosis: 56 years old
- All Caucasians

20 Controls

- Matched with cases on age, gender and ethnicity

MicroRNA Profiling Analysis

- Exiqon MicroRNA Ready-to-use PCR array was used
- 742 human microRNAs and 6 reference RNAs
- Spike RNAs were used to normalize plate-plate variations

Data Analysis Procedure

748 miRNAs profiled in 40 whole bloods using Exiqon PCR array

209 miRNAs with Ct value <38 in all 40 whole blood samples

52 miRNAs with FC<1.5 & FDR>0.1 in all three case vs. control comparisons.

1) All group: 20 vs.20
2) Breast group: 12 vs. 12
3) Prostate group: 8 vs. 8

Prioritize "housekeeping" miRNAs with small FC & small CV in all three groups.

Step 1: identify miRNAs expressed in all biospecimens

Step 2: identify miRNAs without significant expression changes

Step 3: rank miRNAs based on a composite score of FC (fold change) and CV (correlation of variation)

Prioritization of "Housekeeping microRNAs"

52 miRNAs with FC<1.5 & FDR>0.1 in all three case vs. control comparisons.

1) All group: 20 vs.20
2) Breast group: 12 vs. 12
3) Prostate group: 8 vs. 8

Step 1: using less than 1.2 fold change

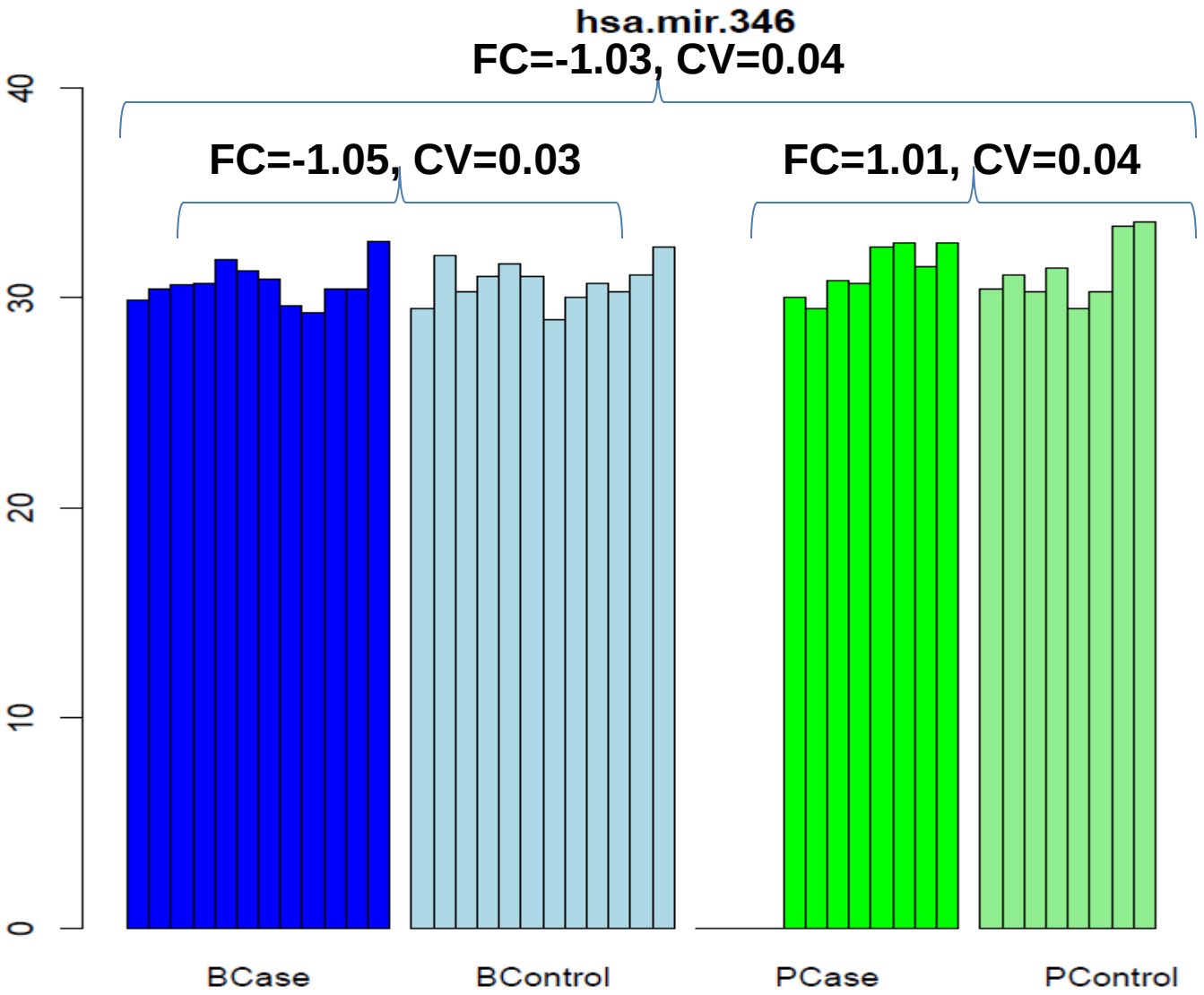
6 miRNAs with < 1.2 Fold in any of the three comparisons

Step 2: smallest CV and fold change

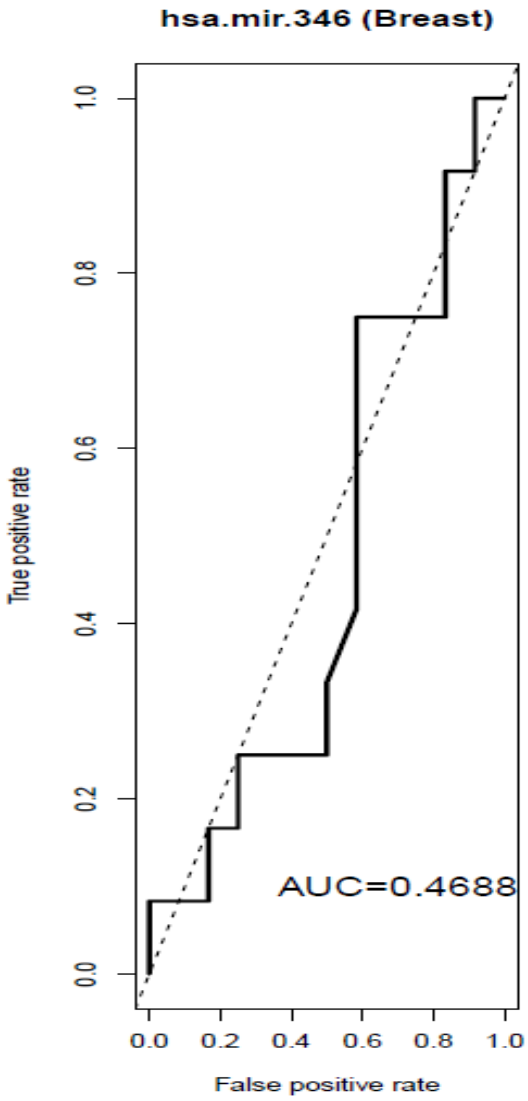
3 miRNAs whose CV rank and FC rank are consistently among the top 10.

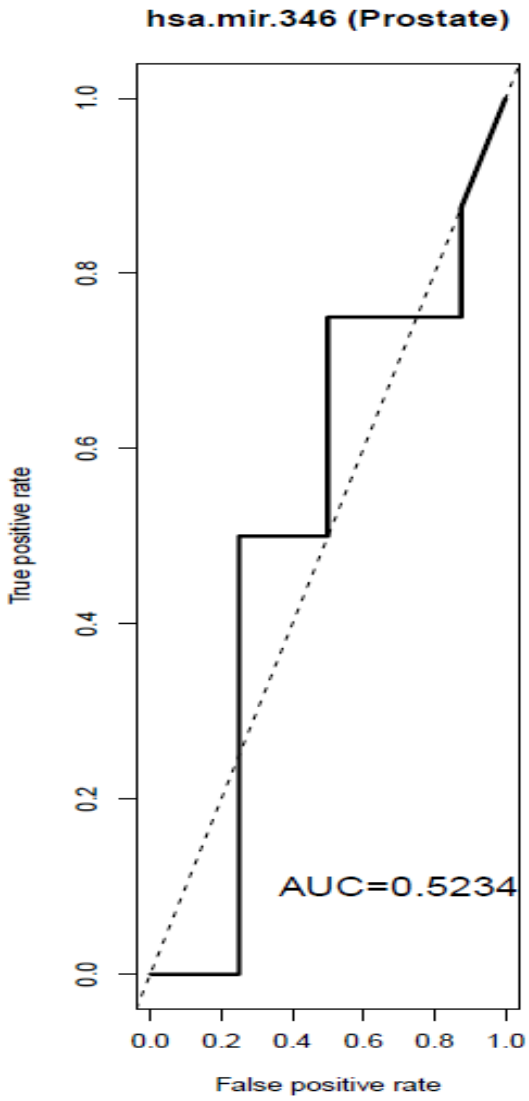
miR-134, miR-346 and miR-934

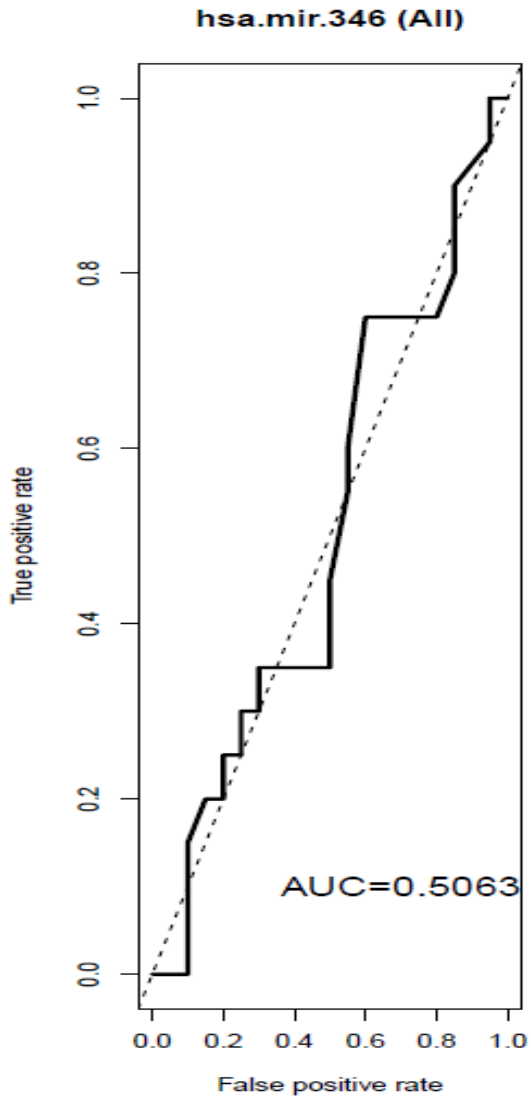
Whole Blood miR-346 Levels Across The Study Subjects



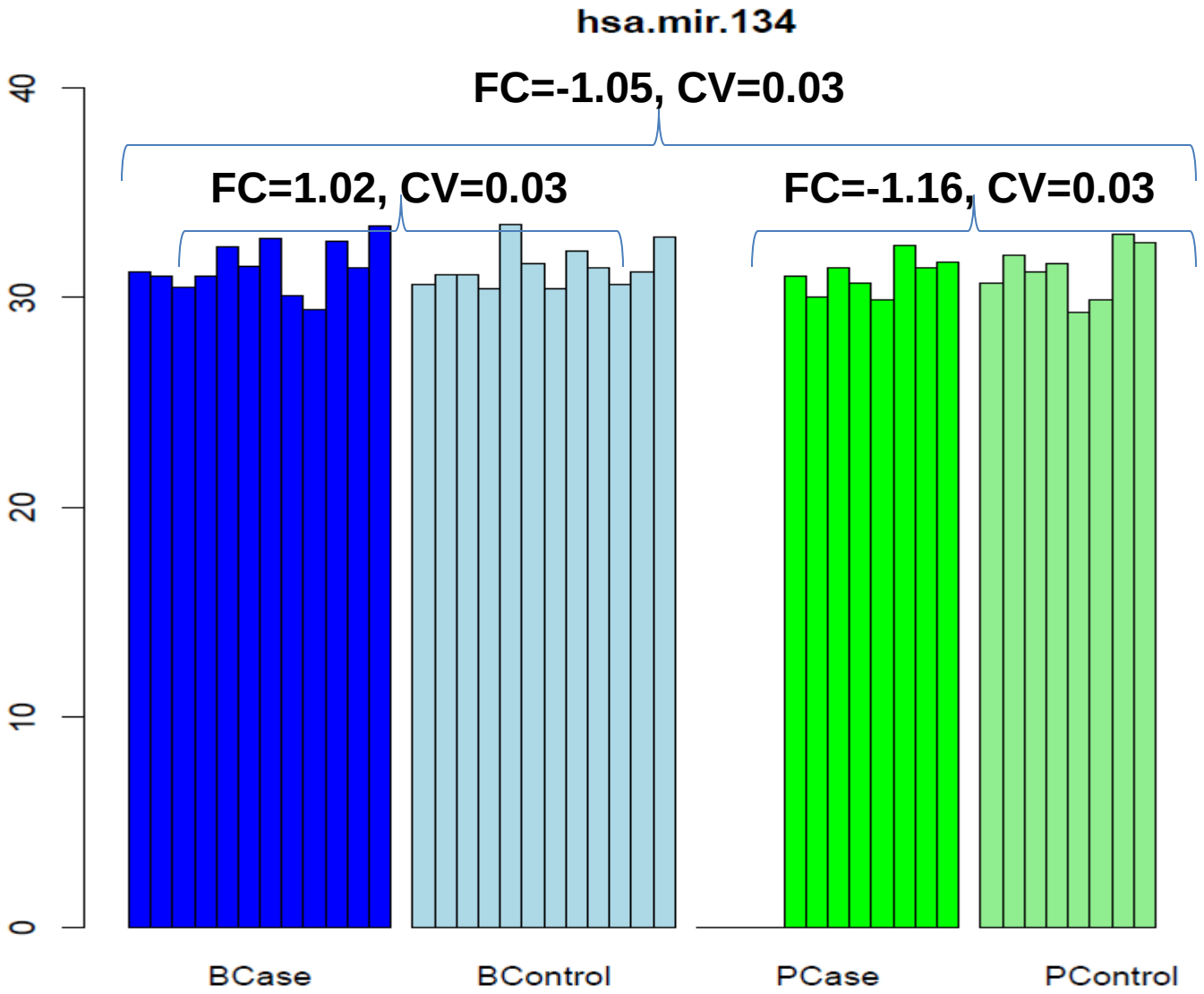
Sensitivity and Specificity Analysis







Whole Blood miR-134 Levels Across The Study Subjects



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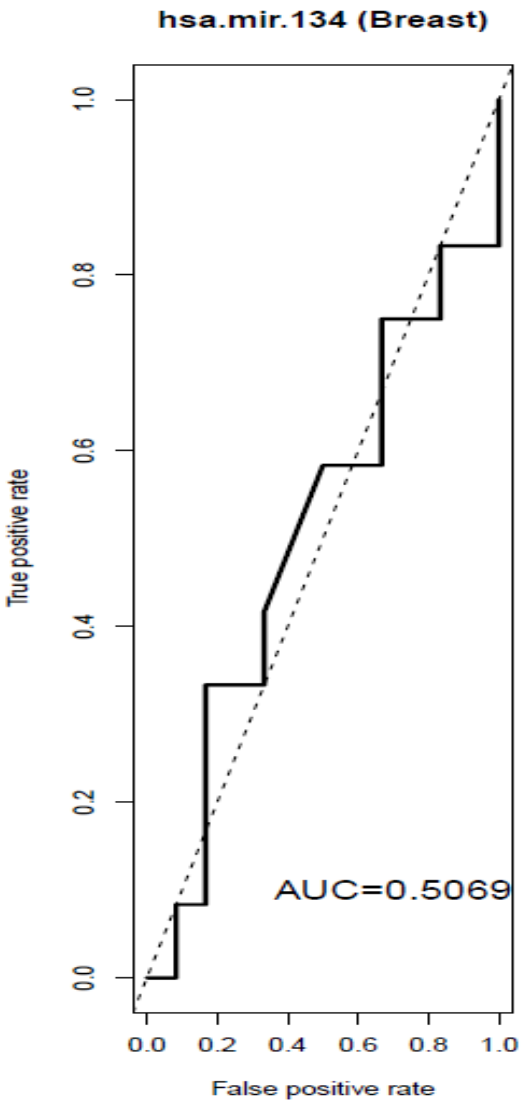
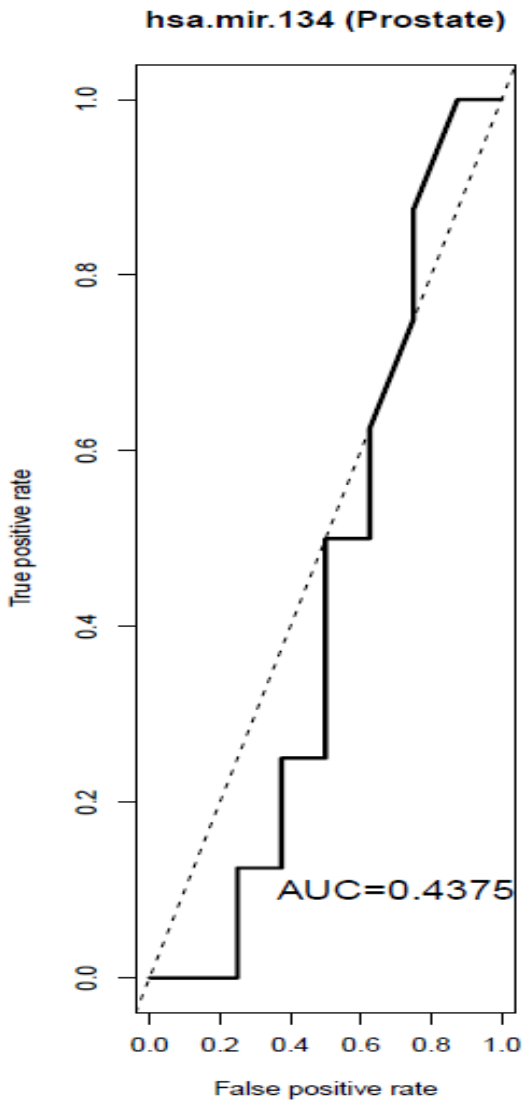
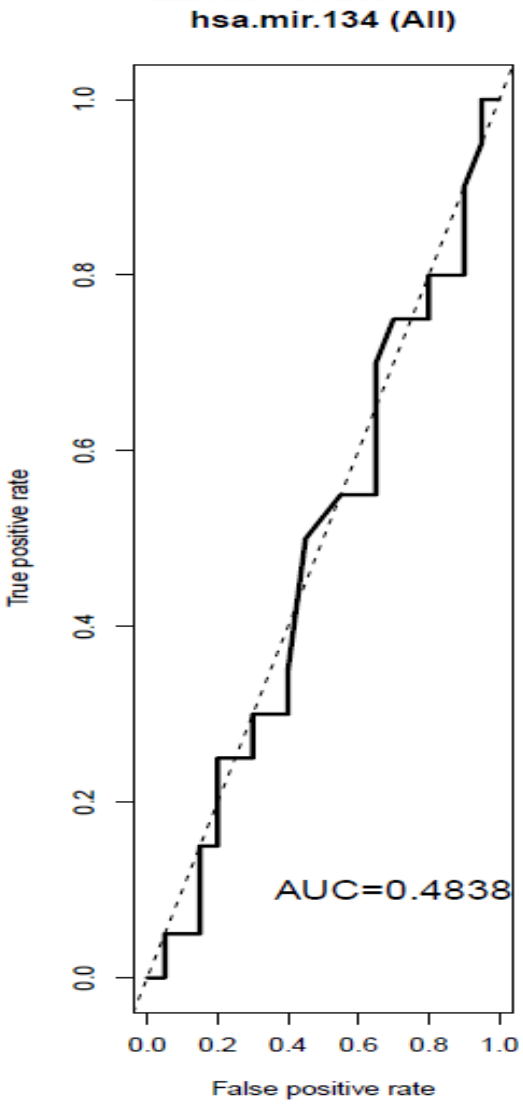
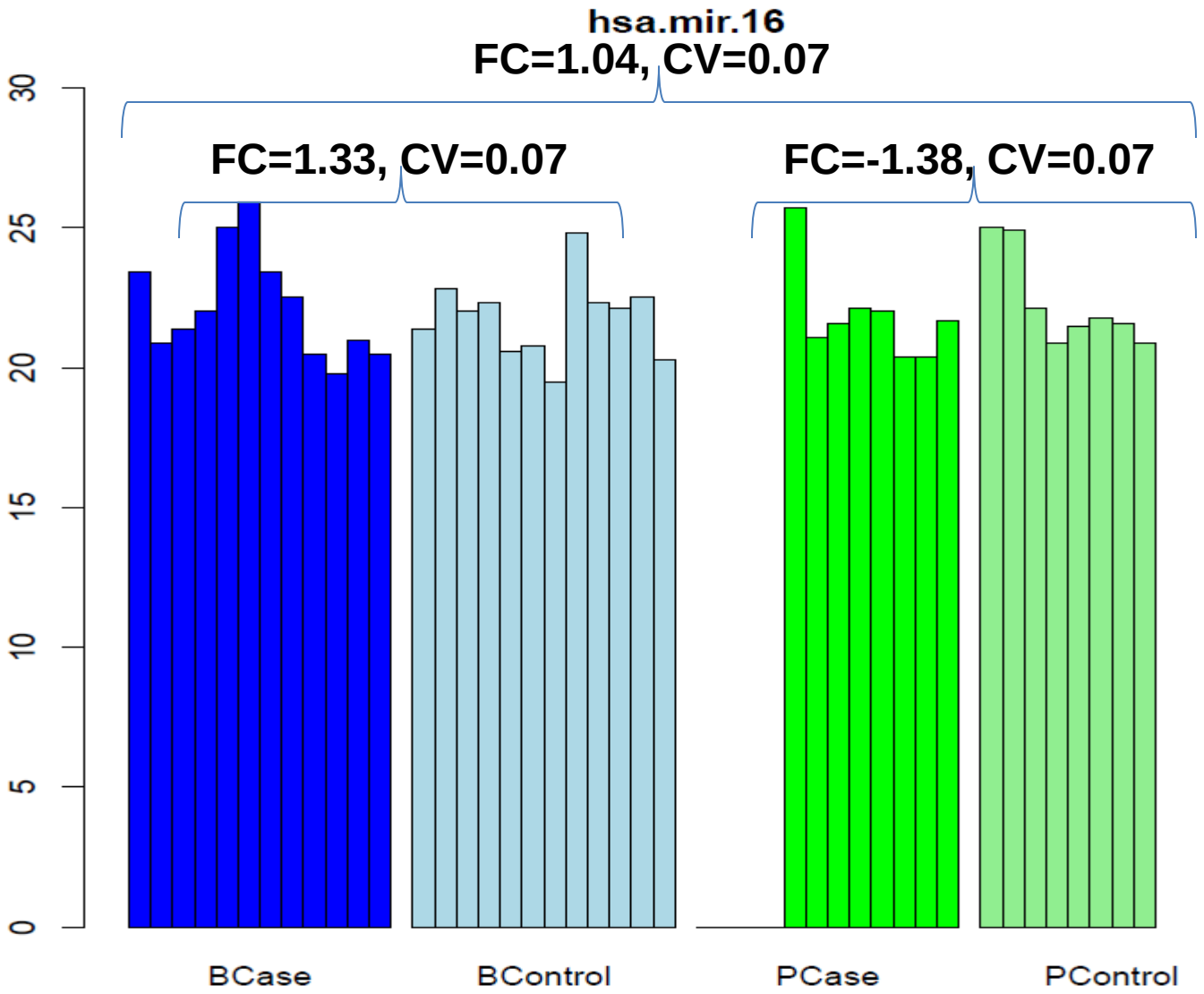


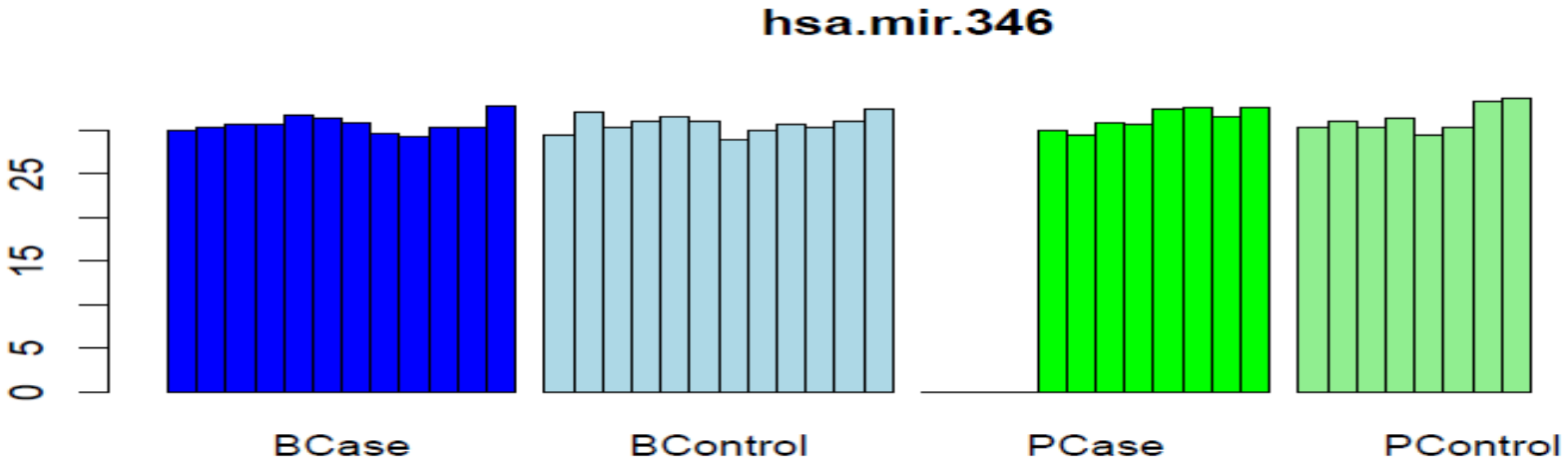


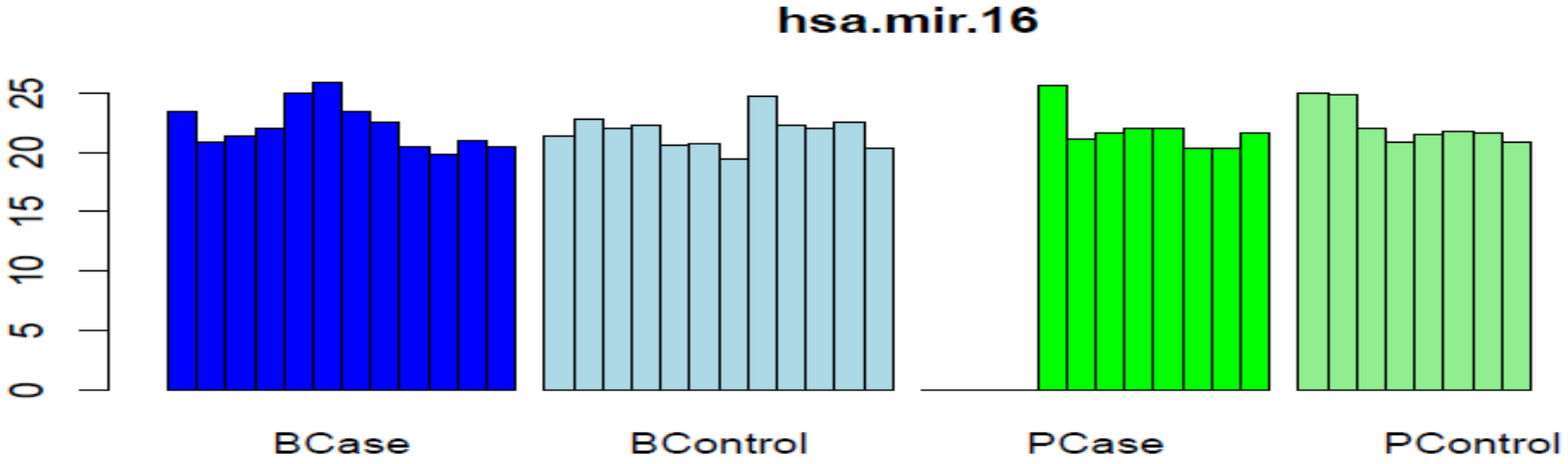


Whole Blood miR-16 Levels Across The Study Subjects

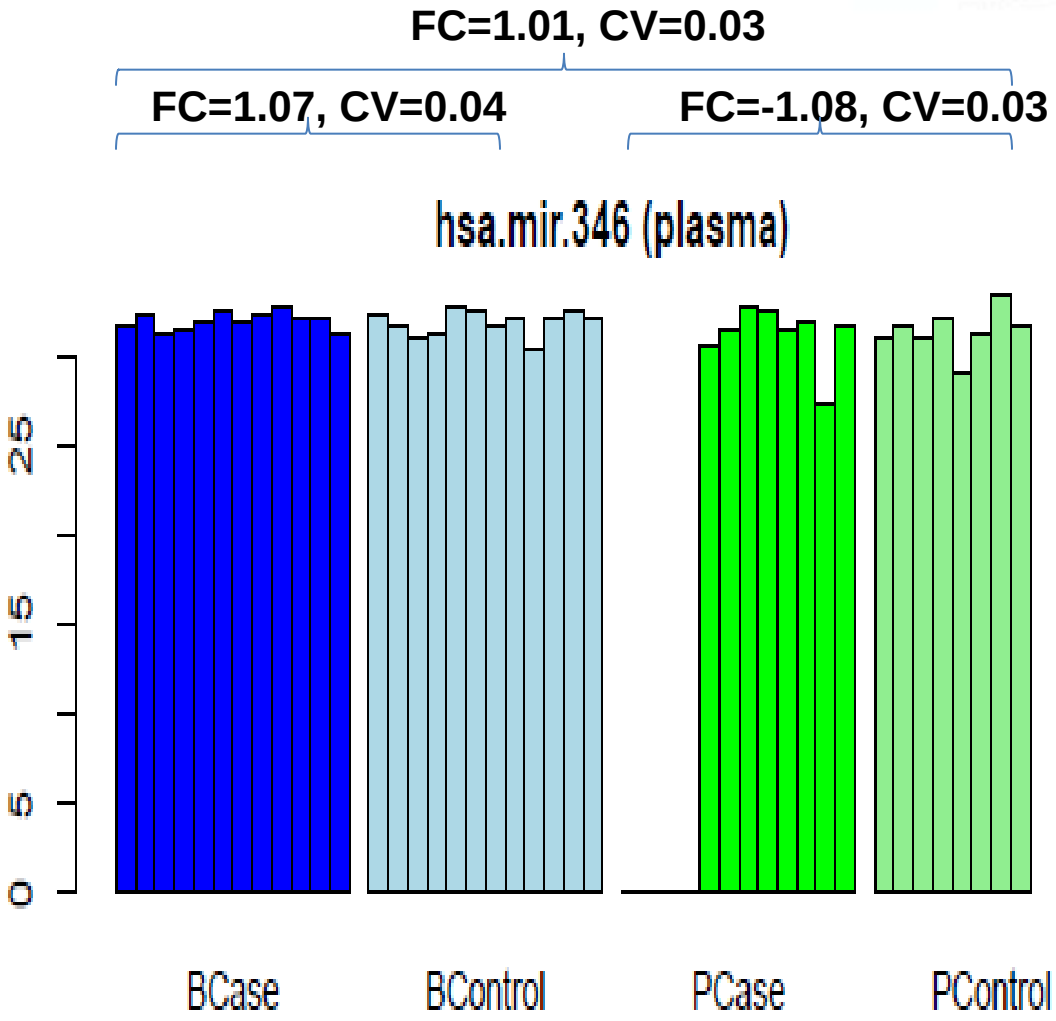


Comparison Between miR-346 and miR-16 Across The Study Subjects





Plasma miR-346 Levels Across The Study Subjects



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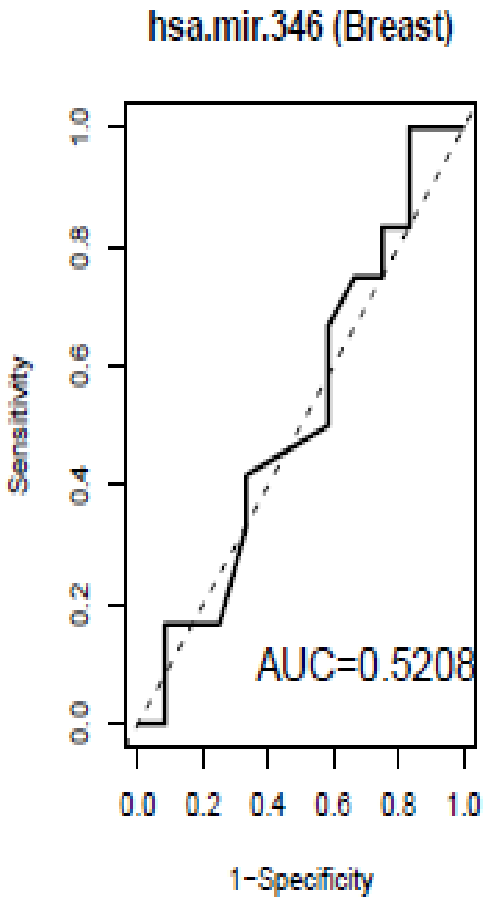

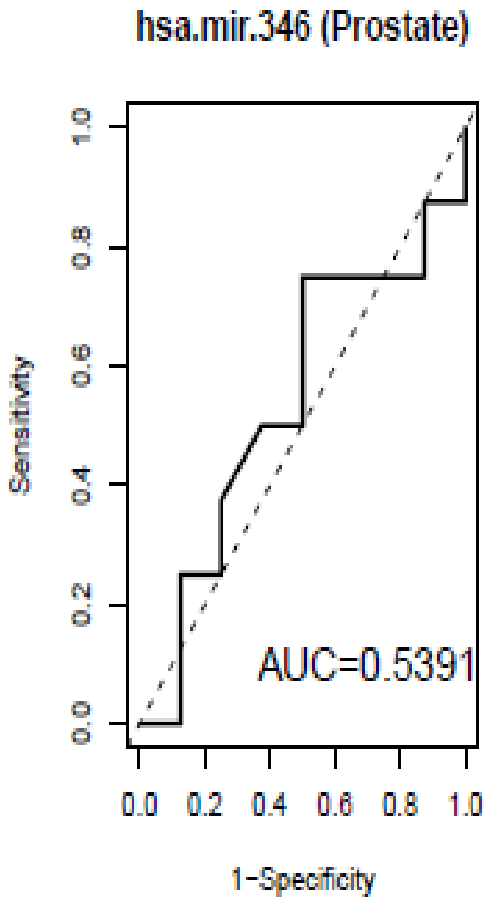

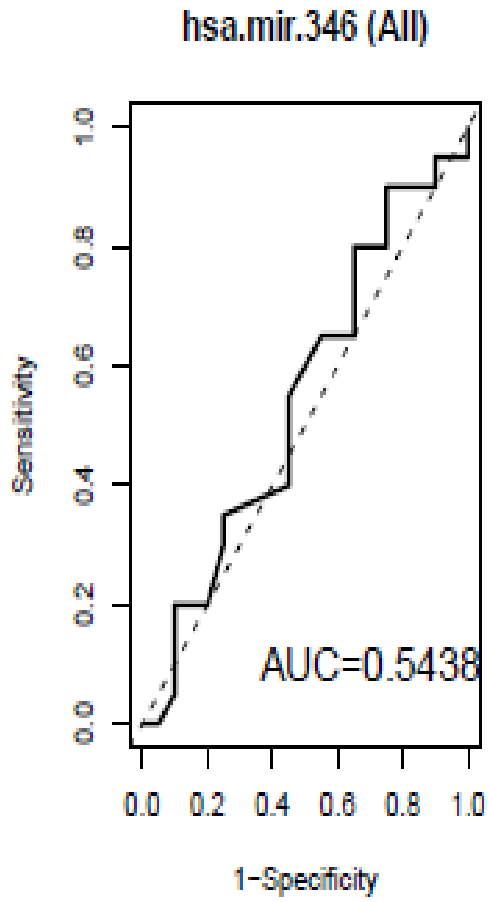

Conclusion

- miR-346, miR-134 and miR-934 are potential candidates for microRNA internal controls in whole bloods.
- miR-346 is a potential candidate for microRNA internal control in plasma.
- The findings will be further assessed in validation analysis.
- If they are validated, these microRNAs will be used to assess the effects of pre-analytic variables on circulating microRNAs.

Acknowledgement

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Thank You!

DBBR Process Flow