miRNA Biomarker Identification Using a Sequencing-Based Nuclease Protection Assay (NPSeq™): Building on EDGE Automation of qNPA™ for RNA Measurement

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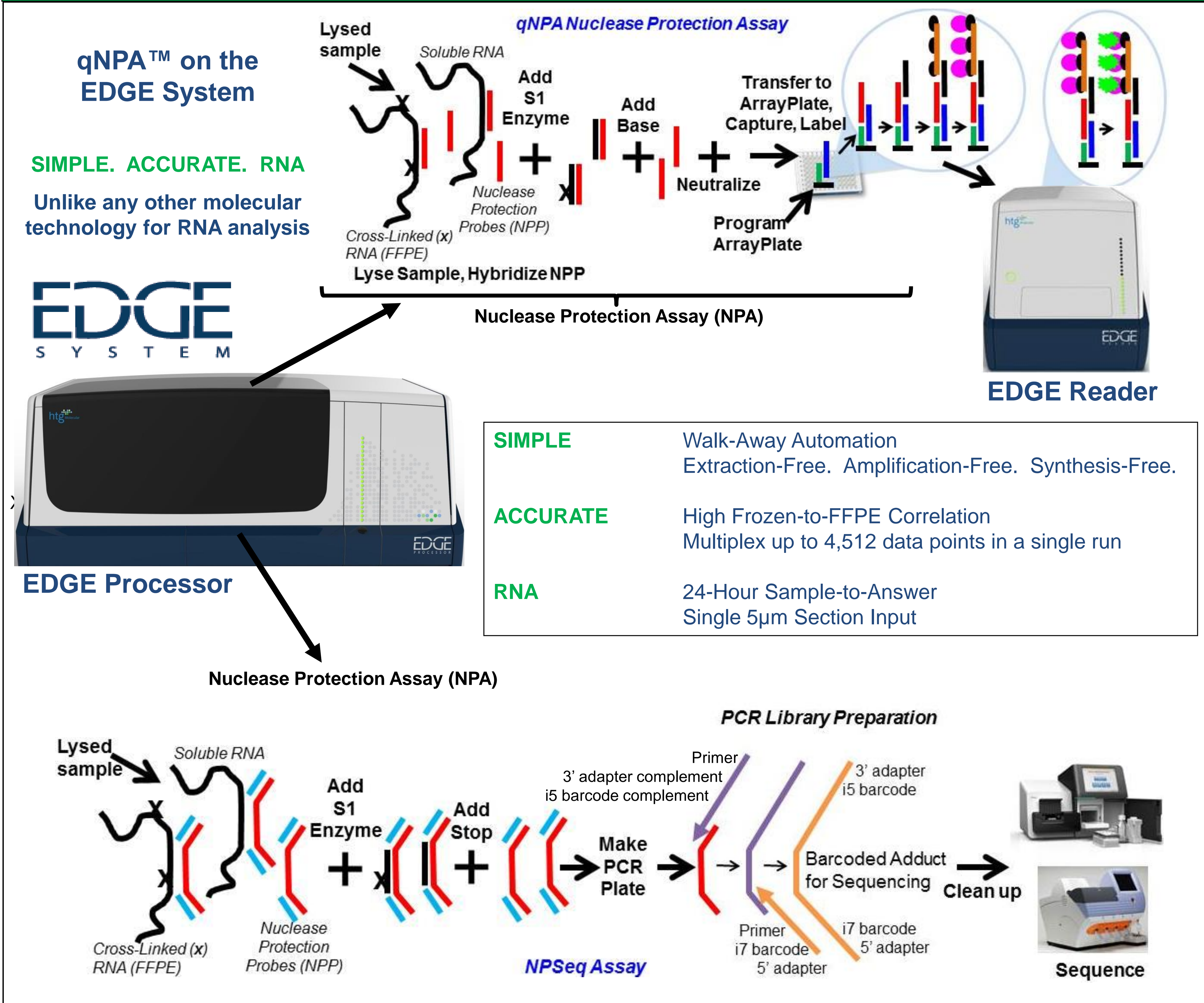
INTRODUCTION

Building on the commercial qNPA™ assay which has been automated on the EDGE System platform marketed by HTG, we developed a novel nuclease protection targeted RNA sequencing assay (NPSeq™) that uses an extraction free lysis process followed by a nuclease protection assay (NPA) to prepare a stoichiometric library of nuclease protection probes (NPP) for measurement. The NPSeq probes are amplified and barcoded by PCR and then sequenced. By barcoding and pooling many samples into a single sequencing run, the sequencing cost/sample can be reduced. Since all the NPP sequences are known, the data analysis is simple and fast. FASTQ files from the sequencer are simply compared to a look-up table of the probes, using the Bowtie short read aligner, and exact matches are counted. NPSeq can be used for biomarker identification in assays of 2,000+ genes. NPSeq retains the advantages qNPA has over other methods for measuring RNA accurately from formalin fixed paraffin embedded (FFPE) tissue to develop and perform assays providing useful additional information[1]. The EDGE System will automate the preparation of samples for NPSeq through the preparation the PCR plate. We have used NPSeq to measure the levels of 1,844 miRNA from miRBase 19 in FFPE brain tissue from deceased normal, Alzheimer's, Parkinson's, and Vascular Dementia patients to identify biomarkers and demonstrate feasibility. Future studies can be performed on the EDGE ArrayPlate assay once biomarkers are narrowed down to a selected few of high interest.

[1]Rimsza et al, *Blood*, 2008 Oct 15, 112 (8): 3425-3433.

qNPA™ AND NPSeq™ ASSAY PROCESS



NPSeq™ PROTCOL AND CONTENT

Sample Preparation Kit and Protocol for Fixed Tissue

- Select samples
  - FFPE, Paraffin embedded FFPE
- Add lysis buffer & denaturation oil from sample prep kit
  - Incubate at 95°C for 10 min
- Add proteinase K from sample prep kit
  - Incubate at 50°C for 60 min
- Transfer lysates to 96 well sample plate

NPA Sample Processing

- Add cocktail of nuclease protection probes (NPP) and incubate
- Add S1 nuclease enzyme and incubate
- Add Stop buffer and incubate

PCR to Prepare Sequencing Library from the NPP

- Add aliquot of each NPA sample to PCR Master Mix
- Add PCR forward and reverse primers with barcodes and adaptor sequences
- Carry out 15 cycles of PCR
- Pool, Gel purify, Sequence

PERFORMANCE

NPP Cocktail QC

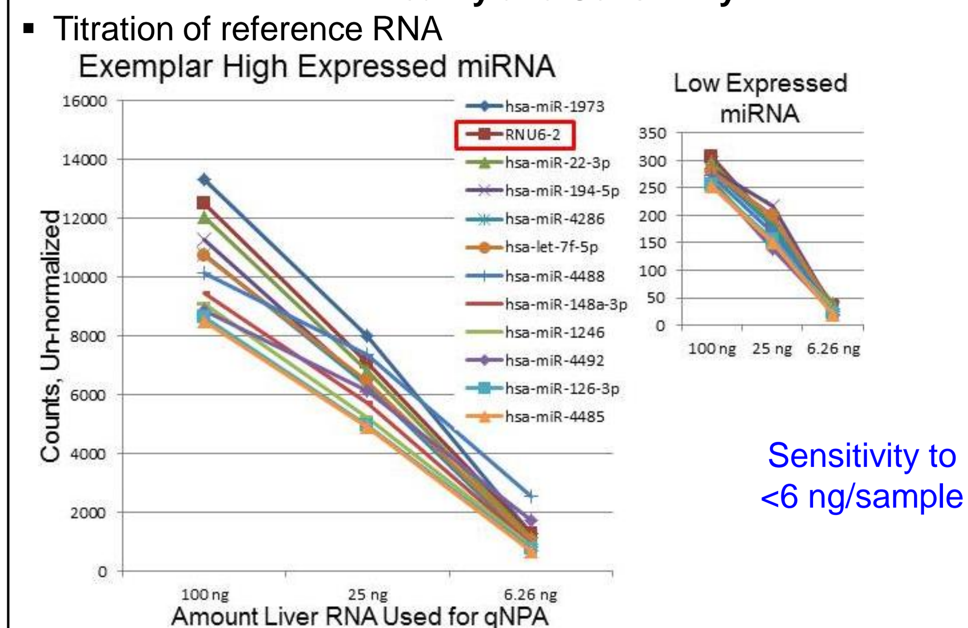
- 1,877 miRNA from miRBase 19 plus 19 mRNA housekeepers and negative control passed QC by sequencing

Inter-Sequencing Run Reproducibility

- 6% average CV between triplicate NPA reactions
  - Genes with expression levels >100 reads,250 ng/sample reference prostate RNA

Linearity and Sensitivity

- Titration of reference RNA



Sensitivity to <6 ng/sample

- Titration of clinical FFPE

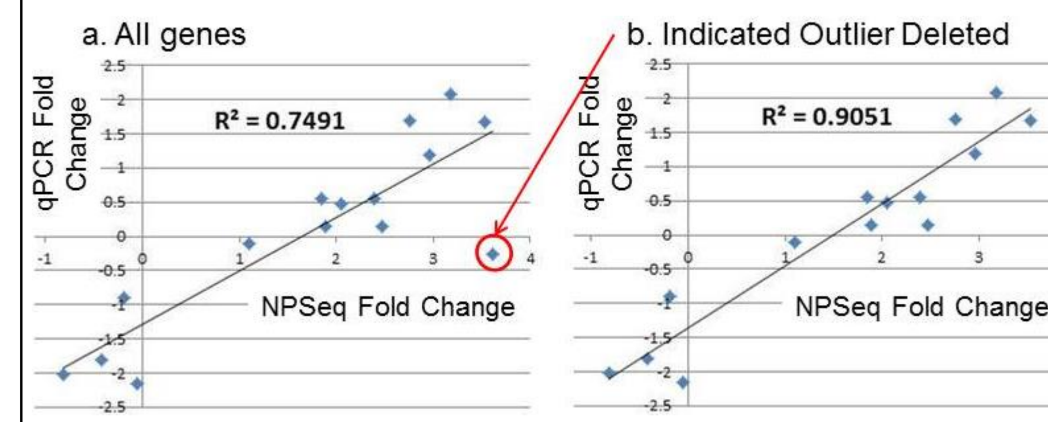
Counts, Un-normalized — Area of FFPE Section Used for qNPA (2 mm2, 1 mm2, 0.5 mm2); hsa-miR-4284, hsa-miR-4286, hsa-miR-3135b, hsa-miR-1260b, hsa-miR-4516, hsa-miR-4488, hsa-miR-1246, hsa-miR-4508, hsa-miR-125b-5p, hsa-miR-3676-5p, RNU6-2, hsa-miR-124-3p

Sensitivity to <0.5mm^2 area, 5 μm thick section

CROSS-PLATFORM PERFORMANCE

Compare NPSeq to qPCR

- Test reference RNA
  - Human prostate total RNA (AM7988), human liver total RNA (AM7960)
  - Compare fold change NPSeq results to qPCR performed on select miRNA

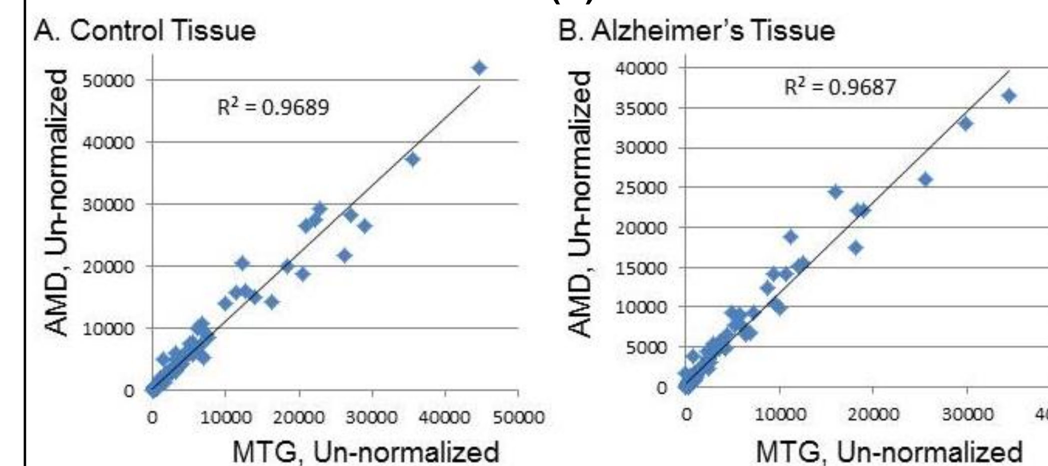


- With Removal of one outlier: $R^2 = 0.905$

RESULTS: SELECTION OF BRAIN REGION

- Compared the middle temporal gyrus of the cerebral cortex (MTG), and subcortical regions of the hippocampus (HIP) and amigdala (AMG)

Exemplar data: AMD vs MTG for Control (A) and Alzheimer's disease (B) tissue



- Identified miRNA that were differentially expressed between regions
  - No large fold change differences observed
- Selected the MTG region for biomarker discovery
  - Most accessible, largest number of Control (Ctr), Alzheimer's Disease (AD), and Parkinson's Disease (PD) and Vascular Dementia (VAD) matched FFPE, frozen and serum samples

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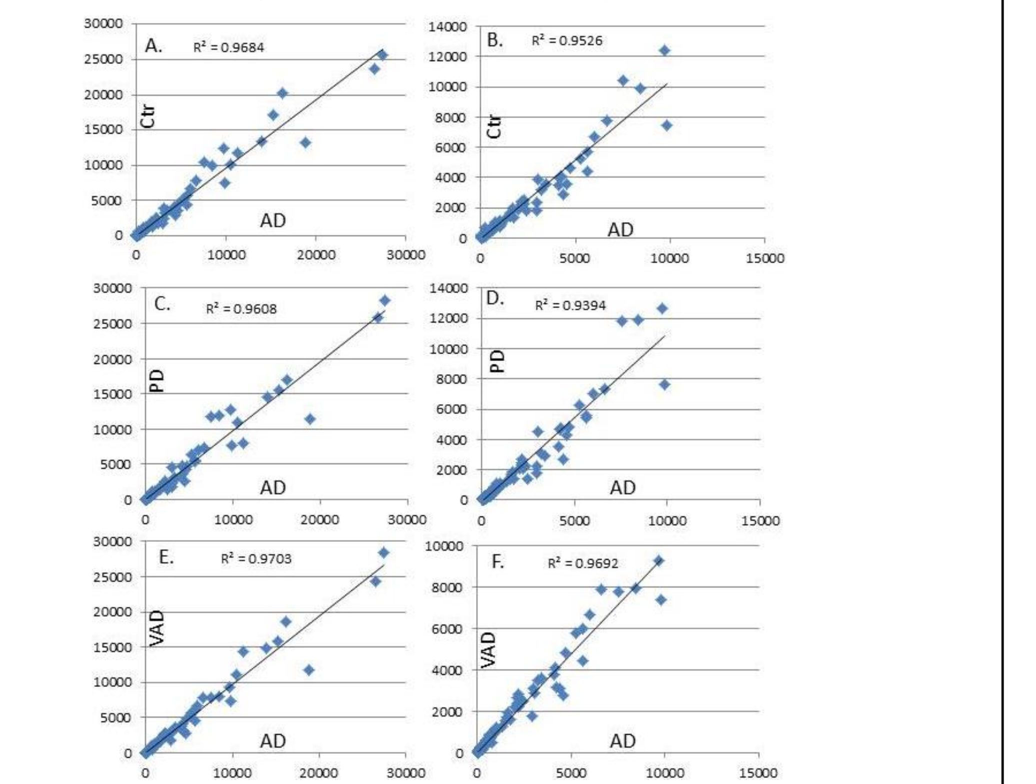
RESULTS: BIOMARKER DISCOVERY

Comparison of 298 miRNA Expressed at >100 Reads

- A: Alzheimer's (AD, horizontal axis) vs Control (Ctr), full scale, all miRNA
  - B: AD vs Ctr, expanded scale, lower expressed miRNA
- C: Alzheimer's (AD, horizontal axis) vs Parkinson's (PD), full scale, all miRNA
  - D: AD vs PD, expanded scale, lower expressed miRNA
- E: Alzheimer's (AD, horizontal axis) vs Vascular Dementia (VAD), full scale, all miRNA
  - F: AD vs VAD, expanded scale, lower expressed miRNA



CONCULSIONS AND NEXT STEPS

NPSeq miRBase 19 Assay

- Assay established, measuring
  - 1,877 of the miRNA in miRBase 19
  - 19 housekeeper mRNA
  - Internal negative control
- High sensitivity: requires low amounts of sample whether FFPE or RNA
- High reproducibility: average 6% CV for separately processed samples
- Low sequencing cost/sample afforded by multiplexing barcoded samples
- Performance of the assay has been characterized
- Utility demonstrated with Alzheimer's biomarker study

Alzheimer's Study

- Biomarkers for Alzheimer's Disease have been identified using the NPSeq miRBase 19 assay
  - Several identified that differentiate AD from Ctr, PD, and VAD
  - Additional identified that differentiate AD from either Ctr, PD, or VAD

Next Steps

- Confirm biomarkers and extend study
  - Confirm on a larger independent cohort of patient samples
  - Extend study to measurements of miRNA biomarkers in serum
- Simplify protocol (e.g. eliminate library gel purification step)
- Establish a mRNA biomarker identification assay
  - Surrogate whole transcriptome assay measuring 2600 genes in development

Learn more about the EDGE System

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