BIOBANKING AS PART OF THE PROSTATE CANCER BIOREPOSITORY NETWORK (PCBN): A FOCUS ON DNA, RNA AND PROTEIN DERIVATIVES FROM RADICAL PROSTATECTOMY SPECIMENS

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INTRODUCTION

➢ The Prostate Cancer Biorepository Network (PCBN) is a collaboration between Johns Hopkins and New York University, and is funded by the Department of Defense (DOD).

➢ The goal of the PCBN is to develop a biorepository with high-quality, well-annotated specimens obtained in a systematic, reproducible fashion using optimized and standardized protocols.

➢ We describe our efforts to develop Standard Operating Procedures (SOPs) for the extraction and biobanking of DNA, RNA and protein derivatives from frozen tissues harvested from radical prostatectomy specimens including:

- Side-by-side comparison of extraction methods/optimization for prostate tissues.
- Establishing standardized methods for assessing sample quantity and quality.
- Development of routine real-time PCR assays for DNA and RNA samples based on both housekeeping genes (GAPDH, 18S, β-globin) and markers differentially expressed in prostate cancer (Racemase, Hepsin) that are performed on all samples included in the biorepository.

➢ Additional efforts currently underway include determining global changes in RNA expression and protein quality in frozen tissues collected from open radical prostatectomy versus laparoscopic prostatectomy and the development of SOPs for DNA/RNA extraction from archival formalin-fixed paraffin-embedded prostate tissues.

SOP DEVELOPMENT

➢ Specimen types available from the PCBN include tissue microarrays (TMAs), formalin-fixed paraffin-embedded (FFPE) tissues, body fluids (serum, plasma, buffy coat, prostatic fluid), and DNA derived from seminal vesicle. In addition, samples currently available in the PCBN include frozen prostate tissue-derived molecular derivatives such as DNA, RNA and protein.

➢ The goal in the development of DNA and RNA extraction SOPs was to choose the processing method that preserves the greatest number of analytes while maximizing quality/yield:

DNA

- Method must provide DNA of adequate quality for advanced technologies (e.g., next generation sequencing, exome sequencing, methylation and other epigenetic studies, etc.)
- DNA should be treated with RNase
- Samples must remain free of PCR inhibitors
- **DNeasy Blood and Tissue Kit (Qiagen)**

RNA

- Method must provide RNA of adequate quality for advanced technologies (e.g., microarray, RNA-seq, NanoString, etc.)
- Preservation of small RNAs (microRNA, etc.)
- Samples must remain free of protein, DNA carry-over, PCR inhibitors, etc.
- **Trizol (Invitrogen)**

➢ We conducted a study to determine the amount of frozen tissue to be used for DNA extraction without exhausting the frozen tissue block (**Figure 1**). The number of frozen sections in a tube versus nucleic acid yield was tested and 50 sections per tube was selected as the optimal amount.

➢ The following standard QC methods were established to assess the integrity of the isolated nucleic acid:

- Quantifications using Nanodrop and Agilent Bioanalyzer were compared and examined for inconsistencies (**Figure 2**). Results led to selection of Nanodrop for quantifying RNA and DNA. Additionally, Bioanalyzer will be used to obtain RNA integrity number (RIN).
- Real time PCR protocols were optimized using control DNA and RNA templates to yield PCR efficiency in the range of 90-110% with primers amplifying the housekeeping genes β globin and 18S for DNA and GAPDH and 18S for RNA (**Figure 3**).
- In addition, real time PCR protocols were optimized for biomarkers such as hepsin and racemase that are differentially expressed in prostate cancer.

➢ As part of a study that will examine global changes in RNA and protein expression between frozen tissue collected from open radical prostatectomy and robot assisted laparoscopic prostatectomy, we collected preliminary data on hepsin and racemase expression levels in RNA extracted from the two tissue types (**Figure 4**).

➢ Additional efforts included analyzing the effects of freeze/thaw on RNA quality and amplifiability when stored in nuclease free water versus RNAsecure resuspension solution (Ambion) (**Table 1**).

RESULTS

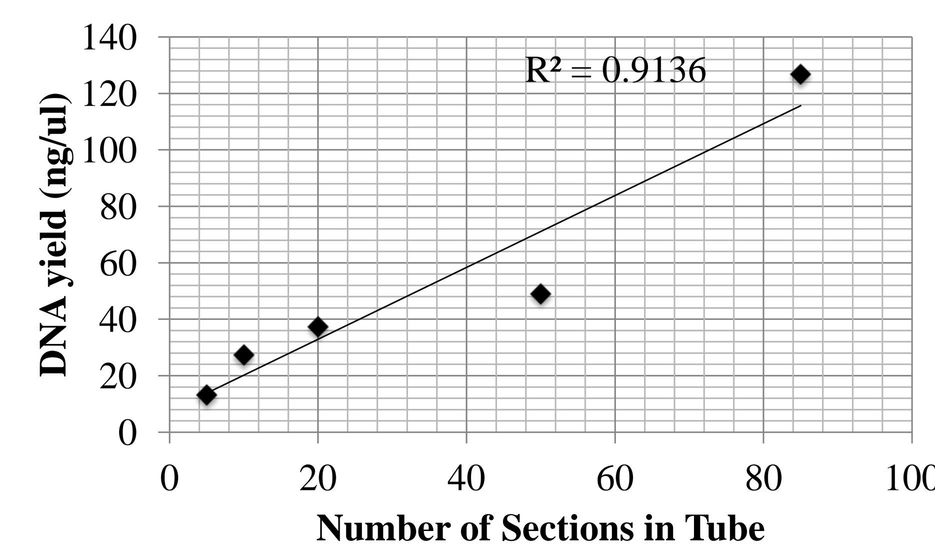

Figure 1. Testing number of frozen sections in tube versus nucleic acid yield.

A)

RNA ID	RNA (ng/uL) Nanodrop	RNA (ng/uL) Nanodrop (redone)	RNA (ng/uL) Agilent	RNA (ng/uL) Agilent (redone)
748	138.7	141.8	98	220
750	116.8	124.8	0	142
752	101.1	101.2	86	153
754	44.7	46.2	73	64
756	106.4	109.9	26	128
758	63.2	103.9	78	186
760	143.4	137.9	58	237
762	97.3	84	151	81
764	77.4	77.4	49	92
766	63.4	74.6	39	72
768	121.7	137.1	36	53
770	101.9	105.7	73	99
772	144.3	116.3	219	131
774	49	52.1	3	4

B) R² = 0.8002

RNA (ng/ul) Nanodrop 2nd Quantification; RNA (ng/ul) Nanodrop 1st Quantification

C) R² = 0.0488

RNA (ng/ul) Bioanalyzer 2nd Quantification; RNA (ng/ul) Bioanalyzer 1st Quantification

Figure 2. Comparing quantification methods as part of SOP development. **(A)** RNA concentrations in ng/uL obtained from Nanodrop and Agilent Bioanalyzer at two different time points. Correlating two readings taken by **(B)** Nanodrop and by **(C)** Agilent Bioanalyzer.

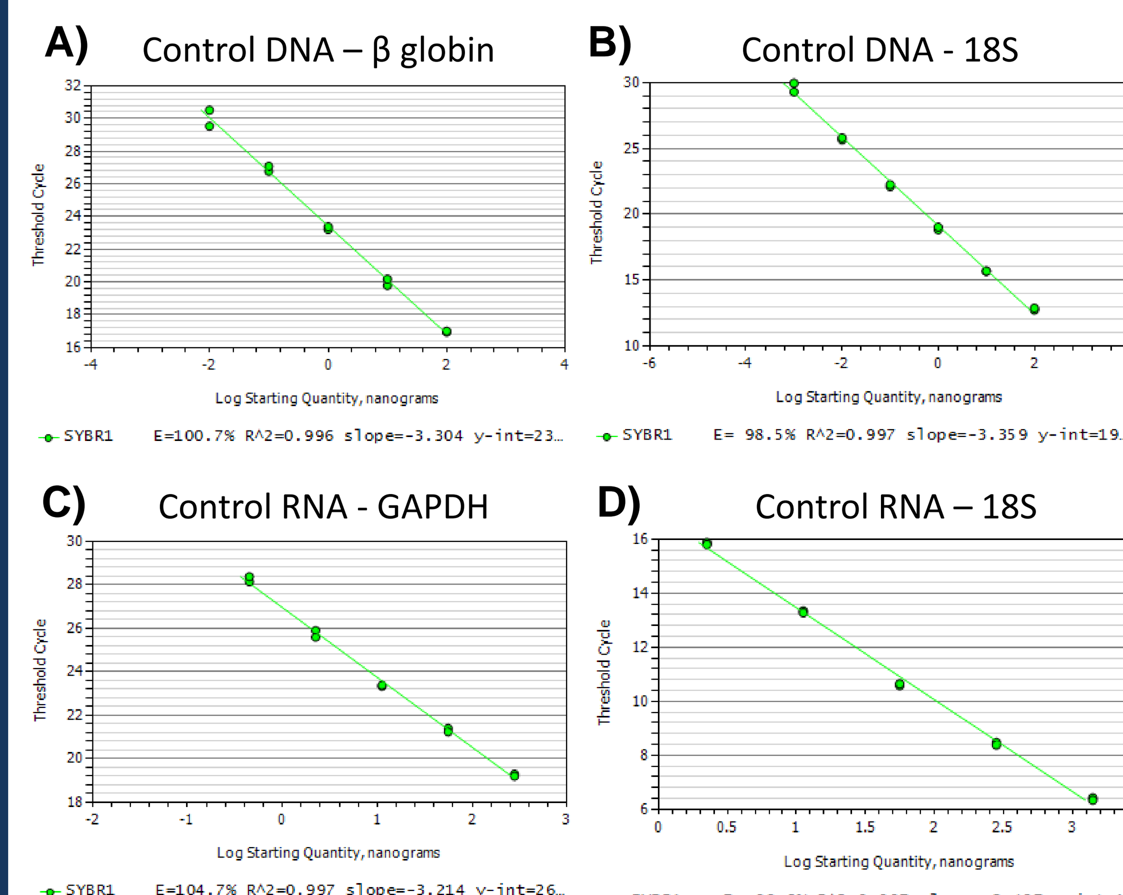

Figure 3. Real time PCR standard curves for amplification of **(A)** β globin and **(B)** 18S from DNA template and for amplification of **(C)** GAPDH and **(D)** 18S from RNA (cDNA) template.

A)

RNA samples (Nanodrop)	Freeze thaw #1 ng/uL (260/280), RIN	Freeze thaw #2 ng/uL (260/280), RIN	Freeze thaw #3 ng/uL (260/280), RIN	Freeze thaw #4 ng/uL (260/280), RIN
LNCaP nuclease free water	145.5 (1.89), 10	146.7 (1.82), 9.8	144.6 (1.88), 9.9	143.1 (1.90), 10
LNCaP RNAsecure	643.1 (2.11), 9.7	631.5 (2.06), 9.8	621.5 (2.15), 9.9	613.7 (2.06), 10
7216 RBP nuclease free water	127.6 (1.76), 8.9	124.0 (1.74), 8.7	120.7 (1.75), 8.9	120.5 (1.76), 9.5
7216 RBP RNAsecure	123.3 (2.34), 9.3	122.1 (2.37), 9.2	119.3 (2.57), 8.7	117.3 (2.14), 9.7
7232 RBA nuclease free water	329.3 (1.88), 8.4	318.7 (1.84), 8.7	323.9 (1.87), 8.8	323.4 (1.88), 8.9
7232 RBA RNAsecure	232.1 (2.14), 9.1	229.0 (2.16), 9.1	226.3 (2.24), 9.2	220.9 (2.08), 9.1

B)

cDNA samples - GAPDH	Ct Mean (freeze thaw #1)	Ct Mean (freeze thaw #4)
LNCaP nuclease free water	18.24	16.84
LNCaP RNAsecure	18.72	15.93
7216 RBP nuclease free water	20.53	18.42
7216 RBP RNAsecure	21.43	18.26
7232 RBA nuclease free water	20.57	19.20
7232 RBA RNAsecure	22.86	22.77

Table 1. Effects of freeze/thaw on **(A)** RNA quantification and quality as determined by Nanodrop readings and RIN number, as well as on **(B)** RNA amplifiability by real time PCR amplification of GAPDH.

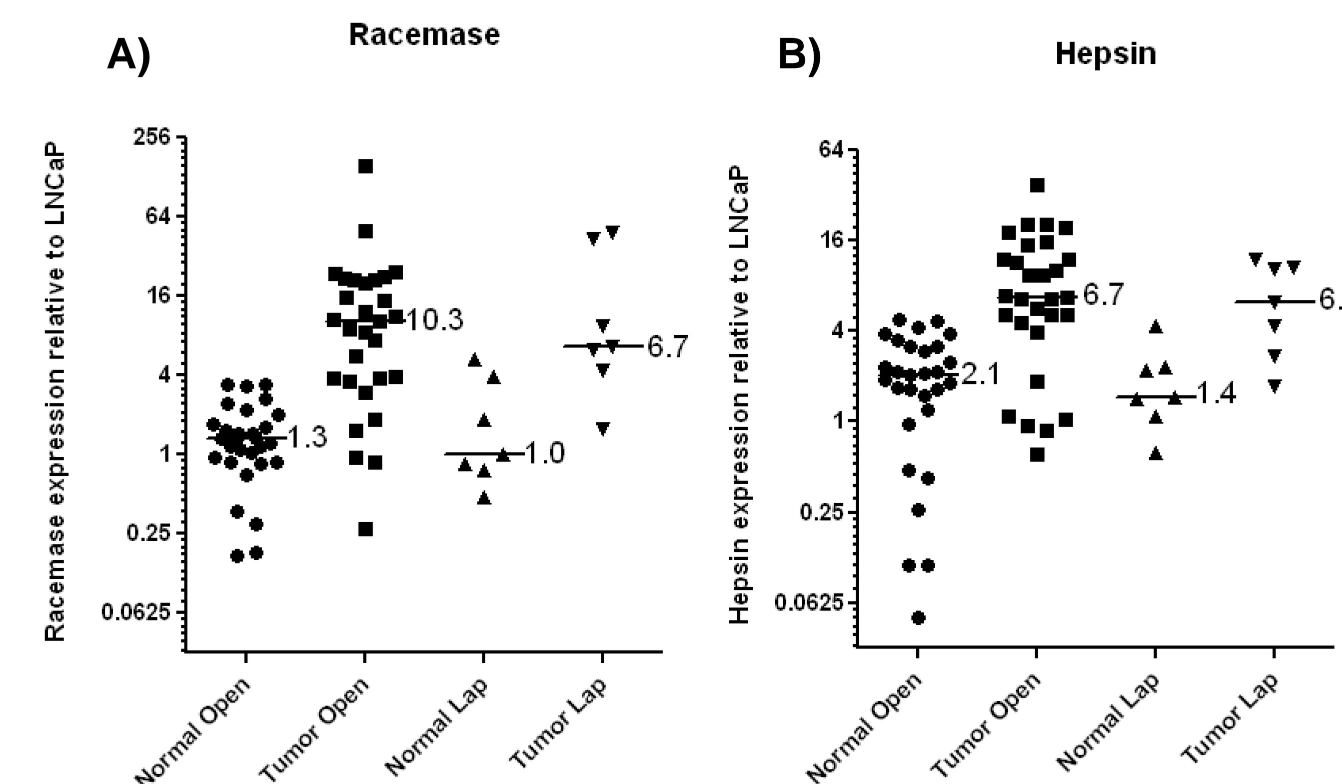

Figure 4. Distribution of the relative expression (Log 2) of **(A)** racemase and **(B)** hepsin as compared to LNCaP among tumor /normal RNA pairs from open radical prostatectomy and robot-assisted laparoscopic prostatectomy. Numbers shown on graphs are median value.

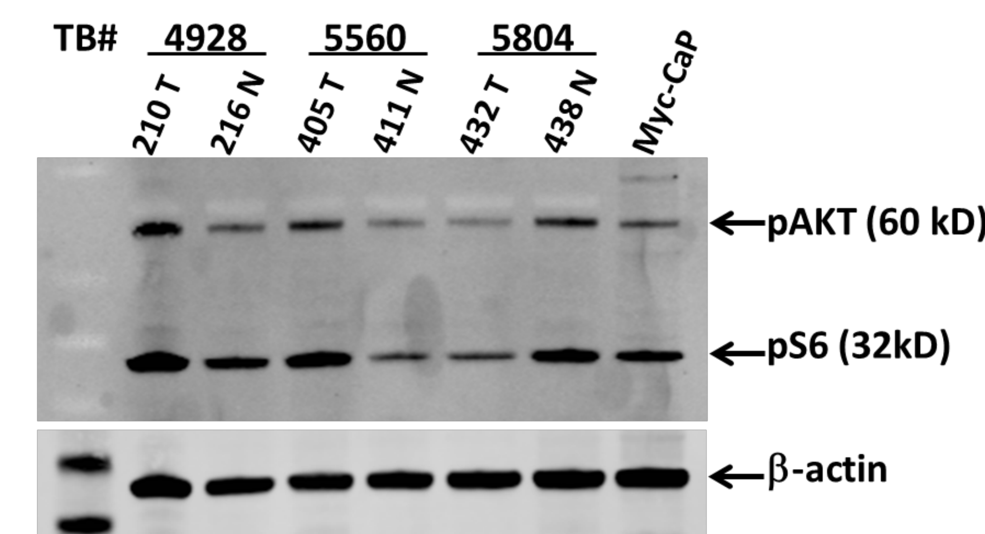

Figure 5. Western blot detection of pAKT and pS6 in protein lysates. Myc-CaP extracts used as postive control and β-actin as loading control.

REPORTABLE DATA ON PCBN SAMPLES

DNA

➢ H&E stained frozen section taken immediately before and immediately after frozen sections collected for sample preparation (scanned slides)

➢ For tumor/normal pairs, H&E sections reviewed for % tumor/normal by a pathologist

➢ Quantification (Nanodrop)

➢ Quantification (real-time PCR for β-globin)

➢ DNA quality (real-time PCR for 18S, β-globin)

RNA

➢ H&E stained frozen section taken immediately before and immediately after frozen sections collected for sample preparation (scanned slides)

➢ For tumor/normal pairs, H&E sections reviewed for % tumor/normal by a pathologist

➢ Quantification (Nanodrop)

➢ RNA quality (RIN number, Agilent Bioanalyzer)

➢ RNA quality (real-time PCR for 18S, GAPDH)

➢ Additional biomarker QC (real-time PCR for hepsin,racemase)

ONGOING/FUTURE EFFORTS

➢ Development of SOPs for protein extraction from frozen prostate tissue including:

- Testing lysis buffers for the extraction of protein and protocol optimization for yield and integrity.
- Yield to be calculated using standard BCA protein assay.
- Subsequent analysis by western blotting for proteins including androgen receptor (AR), cMYC and Hif1α as well as phospho proteins such as pAKT and pS6.

➢ **Figure 5** is an example of a western blot for the detection of pAKT and pS6 in lysates extracted using RIPA buffer.

➢ Development of SOPs for extraction of DNA/RNA from FFPE tissues.

➢ Expansion of sample size for open radical prostatectomy versus laparoscopic prostatectomy experiments. Future analyses with these specimens are expected to include:

- Differences in RNA quality and/or gene expression patterns with age of frozen tissues.
- Global changes in gene expression patterns in RNA extracted from specimens from each surgical technique.
- Suitability of RNA extracted from laparoscopic prostatectomy in advanced RNA assays such as RNA-seq and NanoString assays.

The **Prostate Cancer Biorepository Network** (PCBN) is a public biorepository open to all prostate cancer investigators. Please visit http://prostatebiorepository.org if you are interested in obtaining biospecimens or other services.

ACKNOWLEDGEMENTS

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