
**Radical Prostatectomy, Tissue Microarray
and Diagnostic Biopsy MOP**

Radical Prostatectomy Tissue Block Set

Typical Radical Prostatectomy Block Set Processing Timeline

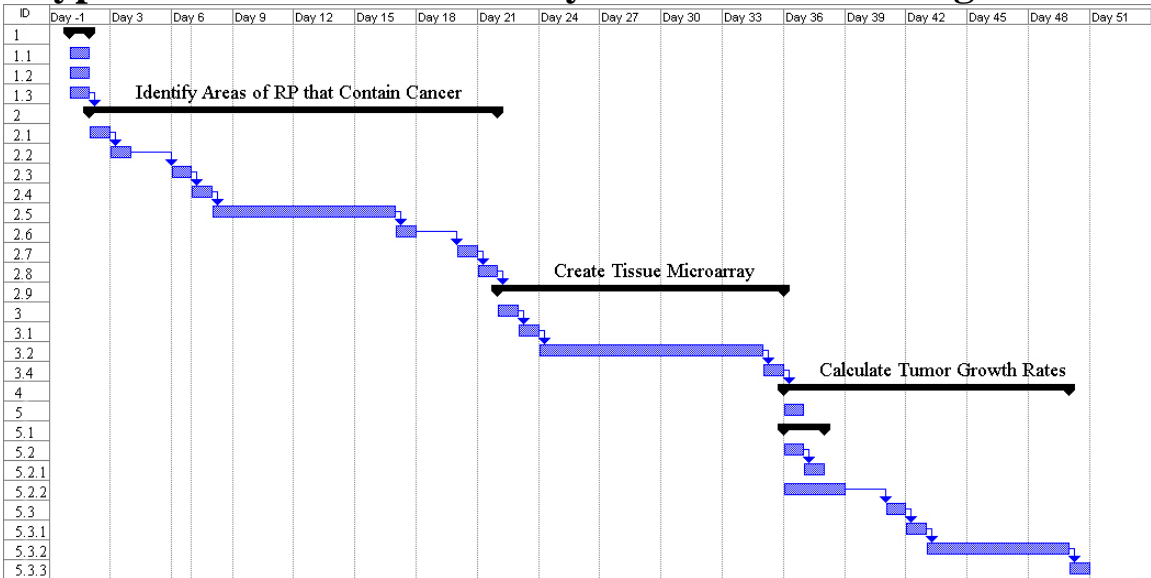


Figure shows the typical life cycle of a patient block set submitted to Core 3. 50 days is average for all sectioning, microarray creation, immunostaining and growth rate calculations.

Radical Prostatectomy Work Breakdown Structure (WBS)

Code	Location	Activity	Deliverables and Notes
1	NC/LA	Receive paraffin blocks from Core 1	Update Citrix server
1.1	NC/LA	Review pathology reports for tissue type	Notify Core 1 of discrepancies
1.2	NC/LA	Generate time frame for return of block	Critical activity
1.3	NC/LA	Log tissue into Core 3 Excel spreadsheet	Generate list of received blocks
2		Identify cancerous regions of tissue	Create H&E staining
2.1	NC/LA	Prepare blocks for microtome sectioning	Begin rehydration 24 hours in advance
2.2	NC/LA	Cut one 4 micron section from each block received	
2.3	NC/LA	Stain each section for H&E	
2.4	NC/LA	Update Excel file to show H&E section created and stained	
2.5	NC	Combine H&E slides from LA and NC	
2.6	NC	Transfer H&E sections to referee pathologist	Pathologist will assign Gleason and encircle regions of interest on each slide
2.7	NC	Retrieve slides from pathologist weekly	
2.8	NC	Digitize slides and archive	
2.9	NC/LA	Cut seven 4 micron sections from each block that will added to the TMA	
3	NC/LA	Create tissue microarray 600 cores (150 cores per quadrant)	Mr Ford will travel to LA for one week every third month to insert LA RPs into TMA
3.1	NC	Determine how many blocks from each block set contain cancer from H&Es	
3.2	NC	Determine coring pattern from number of blocks containing cancer	
3.4	NC/LA	Insert donor cores into recipient block at 0.04mm intervals using a quadrant system of 150 cores per quadrant and record X,Y coordinates	Update Core 3 TMA Excel file
4	NC/LA	Return RP blocks to Core 1 within specified timeframe	Update Citrix server
4.1	NC/LA	UNC RPs are returned directly to UNC hospital by Core 3. All others are returned by student runner to Core 1	UNC pathology signs Core 3 sheet as returned
5	NC	Calculate Tumor Growth Rates	
5.1	NC	Section RP TMA at 6 microns using the	

Radical Prostatectomy, TMA and Diagnostic MOP

Prepared by Harris Ford

September 26, 2007

		instrumedics™ tape transfer method	
5.2	NC	Dual label Immunostain one section for proliferation and apoptosis	
5.2.1	NC	Immunostain for KI-67	
5.2.2	NC	Immunostain for ACINUS	
5.3	NC	Microscopy: Acquire images from cancerous regions	Save images as TIFF files
5.3.1	NC	Transfer images to computer workstation and mask image by removing all stroma and artifacts	
5.3.2	NC	Run masked files through automated analysis program to generate the total number of cells present	
5.3.3	NC	Visually analyze the number of positive cells for proliferation and total number of positive cells for apoptosis	Update Core 3 Excel file
5.4	NC	Transfer data to biostatistician for growth rate calculation	Transfer should be made on the 2 nd Monday of each month

Diagnostic Biopsies and TURPs

Typical Diagnostic Block Set Processing Timeline

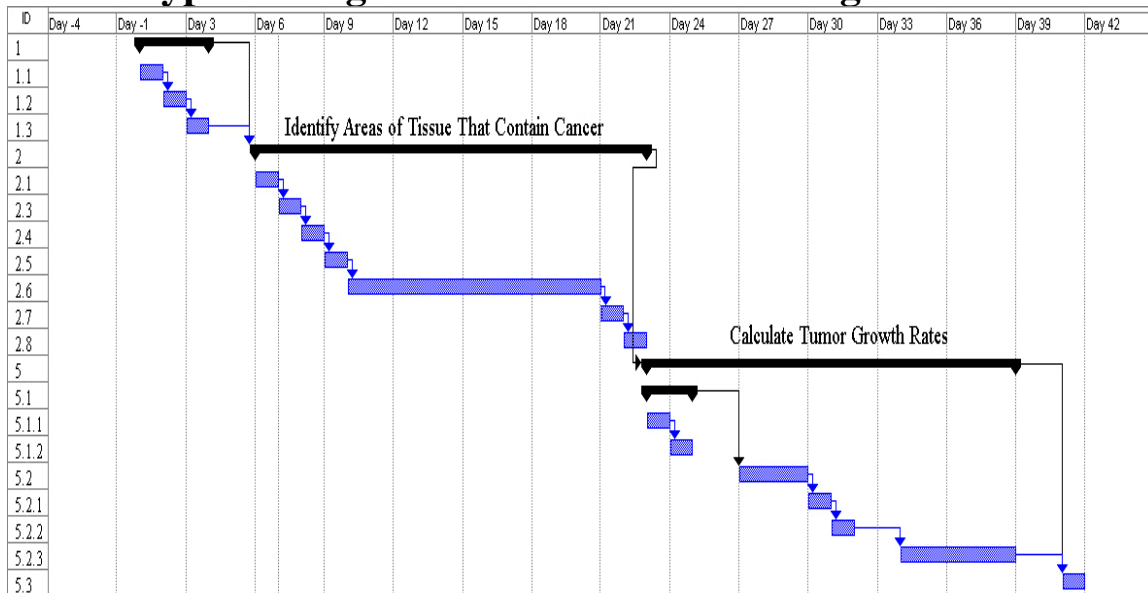


Figure shows the typical life cycle of a patient block set submitted to Core 3. 40 days is average for all sectioning, microarray creation, immunostaining and growth rate calculations.

Diagnostic Biopsy Work Breakdown Structure (WBS)

Code	Location	Activity	Deliverables and Notes
1	NC/LA	Receive paraffin blocks from Core 1	Update Citrix server
1.1	NC/LA	Review path reports for tissue type	Notify Core 1 of discrepancies
1.2	NC/LA	Generate time frame for return of block	Critical activity
1.3	NC/LA	Log tissue into Core 3 Excel spreadsheet	Generate list of received blocks
2		Identify cancerous regions of tissue	Create H&E staining for referee pathologist to assign Gleason Grade and identify cancerous regions of tissue
2.1	NC/LA	Prepare blocks for microtome sectioning	Begin rehydration 24 hours in advance
2.2	NC/LA	Cut seven 4 micron section from each block received	
2.3	NC/LA	Stain one section for H&E	
2.4	NC/LA	Update Excel file to show H&E section created and stained	
2.5	NC	Combine H&E slides from LA and NC	
2.6	NC	Transfer H&E sections to referee pathologist	Pathologist will assign Gleason and encircle regions of interest on each slide
2.7	NC	Retrieve slides from pathologist weekly	
2.8	NC	Digitize slides and archive	
4	NC/LA	Return Biopsy and TURP blocks to Core 1 within specified timeframe	Update Citrix server
5	NC	Calculate Tumor Growth Rates	
5.1	NC	Dual label Immunostain one section for proliferation and apoptosis	
5.1.1	NC	Immunostain for KI-67	
5.1.2	NC	Immunostain for ACINUS	
5.2	NC	Microscopy: Acquire images from cancerous regions	
5.2.1	NC	Transfer images to computer workstation and mask image by removing all stroma and artifacts	
5.2.2	NC	Run masked files through automated analysis program to generate the total number of cells present	
5.2.3	NC	Visually analyze the number of positive cells for proliferation and total number of positive cells for apoptosis	Update Core 3 Excel file
5.3	NC	Transfer data to biostatistician for growth rate calculation	Transfer should be made on the 2 nd Monday of each month

Radical Prostatectomy, TMA and Diagnostic MOP

Prepared by Harris Ford

September 26, 2007

Full Time Equivalent for Radical Prostatectomy Budget Estimation

Technician time for radical prostatectomy microarray construction based on 9 blocks submitted per patient and a technician dedicating 6 hours per day*

Job function	Unit time required	35 specimen/month = Approximately 1 TMA every 2 months	15 patients/month = Approximately 1 TMA every 4 months
Specimen management and tracking		4 days	2 days
Cutting sections from an average of 9 blocks per patient	30 blocks cut per day	315 slides = 10.5 days	135 slides = 4.5 days
H & E Staining of sections from all blocks	1.5h/20 sections	24 hours = 4 days	6.8 hours = 1.1 days
Digitize marked H&E sections and transparency creation	150/day	315 slides = 2.1 days	135 slides = 0.9 days
Database construction	10 h/TMA	6.7 hours = 1.1 days	2.8 hours = 0.5 days
Block retrieval and set up	40 h/TMA	4.4 days	2 days
Construction of Microarrays	40 h/TMA	4.4 days	2 days
Immunostaining for KI-67	8 h/TMA	3.6 hours = 0.6 days	1.5 hours = 0.25
Immunostaining for Caspase-3	8 h/TMA	3.6 hours = 0.6 days	1.5 hours = 0.25
Immunostaining for androgen receptor	8 h/TMA	3.6 hours = 0.6 days	1.5 hours = 0.25
Image collection	2.5 days/TMA	1.68 days	0.72 days
Masking image analysis at 1 images per core:	404 images/35 specimens	3.36 days	1.44 days
Image analysis: parameter set-up, analysis and visual confirmation		4.0 days	1.73 days
Total Number of FTE Required per month		41.7 days	18.08 days

* A technician is expected to provide services 75% of each work day that allows for vacations, sick leave, set up, clean up, maintenance of supplies and equipment and errors. Therefore each technician should provide 195 days of service per year or 6 hours per day.

Full Time Equivalent for Diagnostic Biopsy Budget Estimation

Technician time for diagnostic biopsies based on 6 blocks submitted per patient and assuming 3.75 blocks are positive for cancer.

Job function	Unit time required	70 patients/month	30 patients/month
Specimen management and tracking		8 days	4 days
Cutting sections from each block submitted for H&E	30/day	420 slides = 14 days	180 slides = 6 days
H & E Staining of sections from all blocks	1.5h/20 sections	31.6 hours = 5.2 days	13.50 hours = 2.2 days
cut research specimens from paraffin blocks (7 per cancer positive block)	40/day	262 blocks = 6.6 days	112.6 blocks = 2.8 days
Immunostaining for KI-67	30 sections/ 6 hours	262 slides = 8.8 days	112.6 blocks = 3.8 day
Immunostaining for Caspase-3	30 sections/ 6 hours	262 slides = 8.8 days	112.6 blocks = 3.8 day
Immunostaining for androgen receptor	30 sections/ 6 hours	262 slides = 8.8 days	112.6 blocks = 3.8 day
Image collection	20 minutes/slide	14.6 days	6.4 days
Masking image analysis at 5 images per slide:	20 images/hour	65.6 hours = 11 days	28 hours = 5 days
Image analysis: parameter set-up, analysis and visual confirmation		16 days	6.8 days
Total Number of FTE Required per Month		101.6 days	44.6 days

MANUAL OF OPERATION

**DIAGNOSTIC PROSTATE BIOPSIES
RADICAL PROSTATECTOMIES
TISSUE MICROARRAY**

DIAGNOSTIC BIOPSY: COLLECTION, TRACKING AND STAINING PROTOCOL:

Operations:

1. Core 1 sends letters to pathologists requesting biopsy tissue blocks. One month is built in to allow time to receive the blocks. (*time starts when blocks are received*)
2. Core 1 receives the biopsy tissue blocks and diagnosing pathologist's report. A barcoded tracking system label is affixed to the pathologist's report and all blocks from one subject are placed in a paper mailer. An additional label (from generic sheet of laser labels) indicating "UNC" or "LSU" goes on the paper mailer to assist in returning blocks. *TIME: TBD by Core 1*

Citrix Update Core 1: Barcode labels containing the research subject's Subject ID

Use button "Add."

1st screen

Process Date=Enter or pick today's date

SubjectID=ID number from subject tracking system

LSU ONLY - Process Lab=LSULAB, Location=LSULAB

UNC ONLY - Process Lab=UNCFO, Location=UNCFO.

2nd screen

Pick "Tumor blocks LSU/UNC" from dropdown "Add from Template"

Count=1

Meas=Actual number of blocks for the subject

Unit="PCS"

3rd screen

Save

Print twice (once for mailer, once for path report)

3. Core 1 transfers the blocks, pathology report and the PCaP Pathologist's Report/Log (printed from the tracking system) to Core 3 (at UNC) or LSUHSC. *TIME: 1 day*

Citrix Update Core 3: Mailers are scanned when received by UNC Core 3.

Use button "Update."

1st screen

Select task "Update any location."

2nd screen

Scan all mailers or use "Search."

3rd screen

New Location=UNCTMF/LSUTMF

4th screen

Click Finish

4. Core 3 (UNC) or (LSUHSC) will identify all blocks containing cancer using the pathology report. The blocks that contain cancer will be faced as little as possible to preserve the tissue. Seven 4 micrometer sections will be cut and one of these sections will be used for hematoxylin and eosin staining (H&E). One 4 micrometer section will be cut from all additional blocks for H&E staining. Thus, one section from each block will be placed on a slide, stained with H&E, dried overnight and checked for quality. Slides will be labeled with the study number followed by a dash and a suffix indicating the specific block from which the section was collected (the same suffix written in the original tissue block). If the quality of the H&E section causes the block to be excluded by the pathologist or the technician, the corresponding block will be returned to Core 1 and mailed back to the local pathologist.

For diagnostic biopsies, each research subject will have two blocks if left and right lobe biopsies were submitted separately, 6 blocks if each sextant biopsy was submitted separately and as many as 12 blocks if full template biopsies were performed and submitted separately. All H&E sections will be given to the referee pathologist, Urologic Pathologist.

5. The H&E slides created at UNC as well as slides created at LSUHSC are delivered to Dr. Maygarden via UNC Core 3 (UNC-see address below). Core 3 will deliver slides to the referee pathologist along with the pathology reports and PCAP Pathologist's Biopsy Report/Log. *UNC TIME: 1 day; LSUHSC TIME: 1 week that includes mailing time*

Core 3 Mailing Address: Attn: Harris Ford
 UNC Lineberger Cancer Center
 CB # 7295
 University of North Carolina
 Chapel Hill, NC 27599
 Phone Number: 919-843-9756
 Fax Number: 919-966-3015

OF NOTE: If needed, any tumor block can be returned to the local pathologist within 24 hours using FedEx by paging Harris Ford at 919-216-1072.

6. The referee pathologist will encircle and Gleason grade each region of each biopsy that contains CaP (outlining the area of cancer). CaP should be present in 1 (28%), 2 (38%), 3 (17%), 4 (8%), 5 (6%) and all 6 (3%) of sextant biopsies (Tigrani, Urology 54:689-693, 1999). Dr. Maygarden determines those research subjects for whom the blocks should not be used because such research use would exhaust the specimen. Dr Maygarden will

record Gleason primary and secondary grades directly on each H&E section/slides. *TIME: 2-4 weeks*

7. The referee pathologist returns the slides to Core 3. A computer flatbed scanner is used to scan images of the matched H & E sections for archival purposes. *TIME: 1 day*
8. If a decision is made to include a biopsy that was not identified by review of the pathology report, six additional 4 micrometer sections will be cut from the original block and labeled with the original biopsy number and PCaP study number.
9. From the six research sections cut, representing cancer and benign prostate tissue, Core 3 will use three slides for the immunostaining of Ki-67, Caspase-3 and androgen receptor, as described in the following references:
 1. [Smitherman AB, Gregory CW, Mohler JL](#). Apoptosis levels increase after castration in the CWR22 human prostate cancer xenograft. *Prostate*. 2003;57:24-31.
 2. [Singh SS, Qaqish B, Johnson JL, Ford OH 3rd, Foley JF, Maygarden SJ, Mohler JL](#). Sampling strategy for prostate tissue microarrays for Ki-67 and androgen receptor biomarkers. *Anal Quant Cytol Histol*. 2004;26:194-200.
10. Data accumulated by Core 3 will contain the following information that will be transferred to Core 1 as either an Excel or Word file as needed:
 - 1) Research subject study number
 - 2) Block number
 - 3) Tissue identifier (left or right, apex, mid or base, medial or lateral)
 - 4) Benign or cancer (Gleason primary and secondary grades)
 - 5) Percent biopsy involved by tumor
 - 6) All data collected from immunostaining procedures
11. Core 3 transfers blocks to Core 1 for return

RADICAL PROSTATECTOMY: COLLECTION, TRACKING, TISSUE

MICROARRAY CONSTRUCTION AND STAINING PROTOCOL:

Introduction:

The construction of tissue microarrays is a relatively new high throughput technique that allows for the quick analysis of large numbers of research subjects or research subject samples from archival formalin-fixed, paraffin-embedded tissues. Tissue microarray (TMA) technology enables histologic study of many small specimens processed under the same conditions. Serial sectioning of a TMA block can also allow for multiple replicas of the same slide. TMAs can be constructed using multiple or progressive tumors from research subjects to study genetic alterations and can be analyzed using fluorescence *in situ* hybridization, RNA *in situ* hybridization or standard immunohistochemistry. Due to the small sample size that is taken for each core, it is possible that some genetic alterations may be missed for individual research subjects (Moch et al, Adv Anat Pathol, 8:14-20, 2001). However, the intent is a population survey and not to survey each individual research subject.

The TMA construction process involves taking a cylindrical core from a donor block of formalin-fixed, paraffin-embedded tissue and placing it into a recipient block of paraffin. The cores can range in diameter from 0.6 mm to 2.0 mm. We will use 0.6 mm cores for our study so that the recipient block will contain approximately 600 cores in 150 core patterns (quadrants).

Operations:

1. Core 1 sends letters to pathologists requesting radical prostatectomy blocks. One month is built in to allow time to receive the blocks. (*time starts when blocks are received*)
2. Core 1 receives the tissue blocks and diagnosing pathologist's report. A barcoded tracking system label is affixed to the pathologist's report and a paper mailer containing all blocks from one subject. An additional label (from generic sheet of laser labels) indicating "UNC" or "LSU" goes on the paper mailer to assist in returning blocks. *TIME: TBD by Core 1*

Citrix Update Core 1: Barcode labels containing the research subject's Subject ID
 Use button "Add."
 1st screen
 Process Date=Enter or pick today's date
 SubjectID=ID number from subject tracking system
 LSU ONLY - Process Lab=LSULAB, Location=LSULAB
 UNC ONLY - Process Lab=UNCFO, Location=UNCFO.
 2nd screen
 Pick "Tumor blocks LSU/UNC" from dropdown "Add from Template"
 Count=1
 Meas=Actual number of blocks for the subject
 Unit="PCS"
 3rd screen
 Save
 Print twice (once for mailer, once for path report)

3. Core 1 transfers the blocks, pathology report and the PCaP Pathologist's Report/Log (printed from the tracking system) to Core 3 (at UNC) or LSUHSC. *TIME: 1 day*

Citrix Update Core 3: Mailers are scanned when received by UNC Core 3.
 Use button "Update."
 1st screen
 Select task "Update any location."
 2nd screen
 Scan all mailers or use "Search."
 3rd screen
 New Location=UNCTMF/LSUTMF
 4th screen
 Click Finish

4. Core 3 (UNC) or (LSUHSC) will face each block and minimally cut (to preserve the tissue) a 4 micrometer section for H&E staining. Each section will be placed on slides, stained with H&E, dried overnight and checked for quality. Slides will be labeled with the study number followed by a dash and a suffix indicating the specific block from which the section was collected (the same suffix written in the original tissue block). This will facilitate the identification of the specific block selected for TMA construction. If the quality of the H&E section causes the block to be excluded by the pathologist or the technician, the corresponding block will be returned to Core 1 and mailed back to the local pathologist.
5. The slides are delivered to the referee pathologist (UNC) or mailed (LSUHSC) to Core 3 (UNC-see address below) who will deliver slides to the referee pathologist along with the pathology reports and PCaP Pathologist's Report/Log. *UNC TIME: 1 day; LSUHSC TIME: 1 week that includes mailing time*

Core 3 Mailing Address: Attn: Harris Ford
 UNC Lineberger Cancer Center
 CB # 7295
 University of North Carolina
 Chapel Hill, NC 27599
 Phone Number: 919-843-9756
 Fax Number: 919-966-3015

OF NOTE: If needed, any tumor block can be returned to the local pathologist within 24 hours using FedEx by paging Harris Ford at 919-216-1072.

6. The referee pathologist will encircle and Gleason grade each region of each prostatectomy that contains CaP (outlining the area of cancer). The referee pathologist determines those research subjects for whom the blocks should not be used because such research use would exhaust the specimen. The referee pathologist will record Gleason primary and secondary grades directly on each H&E section/slides. *TIME: 2-4 weeks.*
7. The referee pathologist returns the slides to Core 3. *TIME: 1 day*
8. A computer flatbed scanner is used to scan images of the H & E's for archival purposes.
9. In a pilot study (Singh SS, Qaqish B, Johnson JL, Ford OH 3rd, Foley JF, Maygarden S J, Mohler JL, Anal Quant Cytol Histol 2004;26:194-200.), we found that radical prostatectomy specimens yield an average of 25 blocks per subject with a range of 2- 66. An average of 9 blocks with a range of 2-17 revealed cancer. Radical prostatectomy specimens contained an average of 2 individual cancers (range 1-8). A chart was made of how TMAs should be constructed to capture accurately the mean and variation in Ki-67 (cellular proliferation) and androgen receptor immunostaining.
10. A template of the radical prostatectomy TMA to determine core coordinates is made using Word to act as a guide. Each core location will be described by x,y co-ordinates, research subject study number and Core 3 study number. UNC and LSUHSC samples will be arrayed randomly on the same TMA (to admix state and race). Each transparency is overlaid on the tissue blocks to guide the area of each prostatectomy from which to punch a core. TMAs will be constructed using the 4-quadrant technique. Each quadrant of 150 0.6 mm cores on the array will hold approximately 25 research subjects since each research subject has on average 5 CaP cores included on TMA (Based on sampling strategy) and 1 "normal" tissue core. There are 600 total cores on each block that will include approximately 576 research subject cores and 12 "markers"(mouse lung) to determine proper orientation and 12 internal controls (rat liver). Individual quadrants on the TMA can be completed in isolation to decrease turn-around time for

prostatectomy blocks that must be returned to pathologists within 3 months. Partial TMA completion may be required during start-up due to gradual subject accrual. *TIME: 2 weeks*

11. Data accumulated by Core 3 will contain the following information that will be transferred to Core 1 as either an Excel or Word file as needed:
 - 1) Research subject study number
 - 2) Research Block number
 - 3) Punch identifier
 - 4) Benign or cancer (Gleason primary and secondary grades)
 - 5) Percent prostatectomy specimen involved by tumor
 - 6) TMA block number (for radical prostatectomy TMAs)
 - 7) TMA location (x, y-coordinates) (for radical prostatectomy TMAs)

12. All H&E slides from study tumor blocks and each TMA block will be archived and stored in Core 3 (at UNC).

Citrix Update Core 3: LSU mailers are scanned after TMA is created

Use button "Update."

1st screen

Select task "Update any location."

2nd screen

Scan all mailers or use "Search."

3rd screen

New Location=LSULAB

4th screen

Click Finish

Mailers must then be transferred to the LSU Lab for return to path labs

Citrix Update Core 3: UNC mailers are scanned after TMA is created but before leaving LSU

Use button "Update."

1st screen

Select task "Update any location."

2nd screen

Scan all mailers or use "Search."

3rd screen

New Location=LSUUNC

4th screen

Click Finish

Citrix Update Core 3: UNC mailers are scanned after arriving at UNC

Use button "Update."

1st screen

Select task "Update any location."

2nd screen

Scan all mailers or use "Search."

3rd screen

New Location=UNCCOURIER

4th screen

Click Finish

Mailers must then be transferred to the UNC Field Office (?) for return to path labs

13. Core 3 transfers blocks to Core 1 for return

Citrix Update Core 1: TBD

Use button "Update."

1st screen

Select task TBD.

2nd screen

Scan all mailers or use "Search."

3rd screen

TBD

4th screen

TBD

Click Finish

14. Once the decision is made to include a radical prostatectomy on a TMA, three 4 micrometer sections will be cut and labeled with the original number and PCaP study number. These sections will be stored in case TMA construction proves to be inadequate.

15. TMA construction will proceed as outlined below:

# of RP blocks that contain cancer	# punches made per specimen distributed between blocks containing CaP	# punches made of BPH	Total Punches per patient
1	5	1	6
2	8	1	9
3	9	1	10
4	8	1	9
5	10	1	11
≥6	12	1	13

16. Upon completion of TMA construction, Core 3 will be responsible for cutting the TMA and staining for Ki-67, Caspase-3 and androgen receptor. Core 3 will cut sections as requested by other qualified laboratories. Data furnished to IT and Data Analysis Center will include:

- 1) Research subject study number
- 2) Research Block number
- 3) Punch identifier
- 4) Benign or cancer (Gleason primary and secondary grades)
- 5) Percent prostatectomy specimen involved by tumor
- 6) TMA block number (for radical prostatectomy TMAs)
- 7) TMA location (x, y-coordinates) (for radical prostatectomy TMAs)

Detailed Tissue Microarray Construction Protocol:

A recipient block made with paraffin-containing plasticizers (*J Clin Pathol: Mol Pathol* 2003;56:305–306) will be faced on a microtome and screwed firmly into place in the Beecher Instruments Microarrayer. 0.6mm needles (one for recipient punch and one for donor block punch) are placed in the turret of the instrument and placed in the proper x, y-coordinates. The usable area of a TMA block is about 3 cm x 2 cm. Within this area, x, y-coordinates will be assigned to each punch for each research subject and recorded as described above.

A small core is removed from the recipient block and the turret is moved so that a punch can be taken from the donor block. The punch from the donor block is then placed into the hole previously created. This process can only be done properly using the human eye and requires acquired technical skill.

After all research subjects and controls have been placed into the recipient block, the TMA is warmed at 37°C overnight with a smooth weight placed on top. This allows bonding between the recipient block and the donor cores and flattens the surface. The slide can be separated from the block by immersing it in water and gently tapping it.

The newly created TMA is faced on a microtome and a slide cut for H&E evaluation. Any cores missing will be logged as “lost” and recorded in the database for future reference (up to 20% of cores may be lost).

The Instrumedics Adhesive Tape Transfer system works best in our experience when cutting 0.6 mm TMAs. This system requires that adhesive tape is placed on the microarray block prior to cutting each section. The tape is aligned on an Instrumedic slide that contains a PSA polymer for adhesion. The slides are incubated under UV light to polymerize the paraffin to the slide. After UV incubation, the slides are placed in a TPC solvent to remove the tape. The slides are placed in xylene for 60 seconds and air dried. Finally, the slides are dipped in melted paraffin once and allowed to cool. The TMA sections can be shipped or stored (failure to re-dip the TMA sections resulted in loss of antigenicity).

ACINUS and KI-67 Immunohistochemistry Procedure for *paraffin embedded tissue* using reagents provided with the EnVision Doublestain System

Dual label Immunohistochemistry Protocol for Ki-67 and ACINUS: Briefly: Formalin-fixed, paraffin-embedded prostate biopsies were dewaxed and rehydrated through graded alcohols. Epitope retrieval was performed in citrate buffer (120° @ 21 PSI) for 10 minutes. Tissue sections are blocked for endogenous peroxidase and incubated with anti-KI-67 (MIB-1 clone, DAKO) primary antibody (1:50) for 2 hours at 37°C, amplified using a dextran polymer (EnVision Doublestain System, DAKO) and visualized with diaminobenzidine. The sections are then labeled using an anti-ACINUS primary antibody (1:500) for 1 hour at 37°C, amplified using a dextran polymer and visualized with Fast Red™. Finally, the sections were counterstained using hematoxylin and mounted using an aqueous mounting medium.

Microscopy Using the Leica DRMA2 Microscope and Image Pro Software

Basic Setup:

1. Turn on scope, stage, camera and video monitor. Log on to the PC and start Image Pro software.
2. Under the Acquire menu, start Scope Pro.
3. Load any saved settings if needed.
Under Acquire, start Stage Pro. The program will issue a warning before it initializes the stage. *Make certain that the objectives are out of the path of the stage for this process.*
Choose the radio button that reads “use physical limits of the stage” for the initialization.
4. Ensure that the software is communicating properly with the scope. Turn the objectives such that the 20X lens is in use. In the Scope Pro window, choose the Configure tab and change the Configure Component to Objectives. Do not click OK. Under the Scope tab, click the Update Positions button. At this point, it should recognize that the 20X lens is in position for use.
5. *Important:* Check the LCD on the front of the scope to be sure that you do not see “Set? - ”. This would indicate that no upper or lower stops are set on the automatic stage operation. This would only happen if someone deleted the pre-existing stops set by the Vashaw reps. At any rate, this should be checked at the start of each session, and if need be, stops should be reset (see below).

Light and Video:

1. Set the rheostat dial (on the left side of the scope nearest to you) to 8 V. If this allows too much light for your specimen, you can engage one or both of the neutral density filters. The set of four filters is found on the right side of the scope at the bottom. The neutral density filters are the two farthest from you. The other two filters, from back to front, are the BG-20 (enhances red tones) and the daylight filter (for use with daylight film—removes yellow tonality), and it will be unlikely that you will need to use them.
2. Under the Acquire menu on the main toolbar, choose Set Up Acquire. This will open two windows for the camera: C5810 32 bit, and C5810 control. You may want to check that the dials on the video monitor are in the proper position. To do this, use the C5810 control window and choose the Color Bar icon. When the color bar appears, adjust the brightness such that you can just barely see the white bar that will appear on the right end just below the strip. Check that the dial for contrast is all the way to the right and back just a bit (I'm quoting Jim Leisey here). Then remove the color bar. If your image is still too bright, use the C5810 control window to adjust the exposure time. The setting of 0.03s is closest to real time, so you will not want to adjust far from this setting. Use the C5810 control window to also perform Auto White Balance (making sure the lens is positioned
3. over an area with no tissue). At this point, if specimen is still too bright, re-adjust the rheostat, and white balance again. If the software re-sets the voltage when objectives are changed, reset white balance.

Kohler Illumination:

Note: This process is critical and should be done at the start of each session and each time an objective is changed.

1. On the left side, towards the back of the scope two dials stacked on top of one another. The top dial is used to adjust the aperture (or iris) diaphragm, and the bottom controls the field diaphragm. Focus specimen under the lens. Close the field diaphragm, then the aperture diaphragm. Raise and lower the condenser (silver knob on left side of scope under the stage) until the edges of the octagonal field diaphragm are sharp. Use the two, small silver knobs under the condenser to adjust the position of the octagon such that it is centered in the field of view (looking at video monitor). Open the field diaphragm until the octagon is just out of view.
2. The aperture diaphragm is the more critical of the two settings. This setting controls resolution and contrast of your image, and should certainly be reset on each objective change. To do this, pull out one of the eyepieces on the scope. Look down into the scope where you removed the eyepiece. The dark ring of the aperture diaphragm is visible. Turn the dial to open it until the ring just disappears.

Leica Auto Controls:

1. Focus – The white knob on the right side of the scope is used for both coarse and fine focus adjustments. The stepwise settings are as follows: S0 = 15 nm, S1 =

- 0.1 μm , S2 = 0.7 μm , and S3 = 1.5 μm . Track of which setting is used by checking the lcd on the front of the scope (ie. it will read “S1” if this is what the dial is set to). To change the setting, use the square, black button closest to the dial. Whenever you return to a previously used objective, the scope will reset the stepwise setting to the previous objective.
2. Stage Vertical – The vertical movement of the stage is controlled by the two square, black buttons stacked on top of one another on the right side of the scope. As long as the upper stop is set (check lcd as mentioned previously).
 3. Stage Horizontal – The horizontal movement of the stage is controlled by the joystick unit on the right side of the scope. If you Update Positions under the Scope tab in the Scope Pro window each time you change an objective, the joystick should drive the stage at a speed appropriate for the magnification of the lens. To drive the stage more quickly, press the button on top of the joystick.
 **Note: The stage can also be operated via Image Pro if preferred.

Other Settings:

- Condenser: Set to “H” for brightfield (Use “D” for darkfield, “1” for phase. If doing darkfield or phase, fully open the aperture diaphragm.).
- Filter Cubes: check the lcd to see “BF.” This will indicate if the filter cube setting is appropriate for brightfield. If it is in the wrong position, change using the Image Pro software or use the two stacked, square, black buttons on the *left* side of the scope.
- Setting Stops: There are two square, black buttons just to the right of the lcd on the front of the scope. To delete a pre-existing upper stop, press and hold the upper button. Adjust the stage to appropriate level. To set the stop at this point, press and hold the upper button again. Using the lower button, the same procedure can be done to reset the lower stop. The upper stop should be set using a high mag lens, such as 40X. The position of the upper stop will read on the lcd as “0 μm .” Note that you will still be able to focus past the upper stop. As mentioned previously, the lcd will read “Set? - ” if no stops are set.
- Learn/Change: The two square, black buttons on the left of the lcd can be used to change parfocality. They can also be used to switch the function of the pairs of stacked, square, black buttons on either side of the scope. For instance, you can alter the setting such that vertical stage movement is controlled on the left, and filter cubes are controlled on the right side of the scope. Two vertical, opposing arrows on the lcd will let you know which buttons are currently operating the stage. If you see the arrows on the right end of the lcd, then control of the stage is on the right. Likewise, if the arrows are on the left end of the lcd, then control of the stage is on the left. To switch the functions, press Learn and then press Change as many times as is necessary to highlight the parameter. Press Learn, and then move the focus knob. If it has learned properly, the lcd will flash “EXIT.” To finish, press Learn again. To change parfocality, press Learn and

- then press Change as many times as is necessary to highlight the parameter reading. Change to the objective to set and focus the specimen. Press Learn and then change to the next objective that you want to teach it. Do this for each objective that you are interested in. When parfocality is set, the scope will automatically re-focus when you switch from one objective to the next.
- Correction Collar: The 40X dry objective is fitted with a patented dial that corrects for the thickness of the cover slip. To use this feature, turn the dial all the way to one side and try to focus your specimen. If it is not perfectly sharp, turn back the dial a bit and try again. Keep turning the dial back in the same direction and attempt to focus each time until you find the place where it gives you the sharpest image. This feature is especially helpful in fluorescence.
 - Low Mag: Should you ever use the scope for low magnification microscopy (< or = 2.5X), you will need to flip the lens that sits just above the condenser out of the light path. For all other microscopy, make sure that it is in place. The metal lever that controls it is on the left side close to the condenser.

Image Analysis: Upon receipt of the diagnostic biopsies, sections were cut and stained for H&E (hematoxylin and eosin). These sections were then given to a referee pathologist to identify areas of cancer within the biopsy. The immunostained slides of Ki-67 and ACINUS were compared to these H&E slides in order to localize the regions of cancer. From these regions, images were taken at 40X (Leica) and saved in a TIFF format. The images were masked (stromal areas and artifacts removed) and run through our image analysis system to produce a total cell count for the secretary epithelium. The Ki-67 and ACINUS totals are calculated visual.

References for androgen receptor immunostaining (Project 7):

Ford III, O. H., Gregory, Christopher W., Kim, Desok, Smitherman, Andrew B. And Mohler, James L. Androgen Receptor Gene Amplification and Protein Expression in Recurrent Prostate Cancer 2003

Gregory, Christopher W., He, Bin, Johnson, Raymond T., et al. A Mechanism for Androgen Receptor-mediated Prostate Cancer Recurrence after Androgen Deprivation Therapy 2001