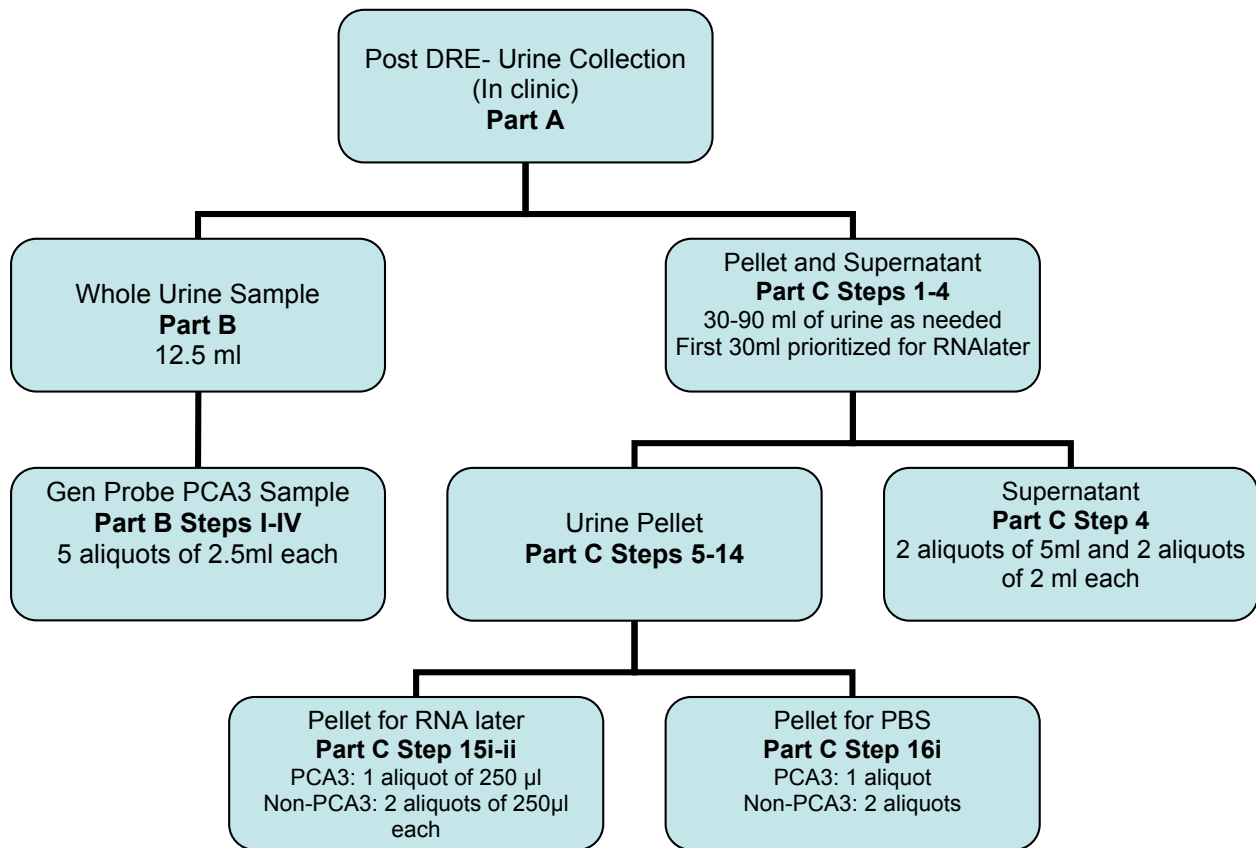


NCI-EDRN Urine Processing Protocol



Urine collection is performed after digital rectal exam (DRE) in the operating room prior to RP.

A. Collection and Transport of Urine to Lab:

Collect post-DRE urine in sterile cup (no preservative) - This is usually provided in the clinic (100 ml). This should be the first 30-60 ml (up to 100 ml) of voided urine (minimum volume 5 ml) following the DRE. If greater than 60ml of urine is collected, the entire volume will be kept. Cool immediately on ice or place into -4°C freezer until ready to transport to begin processing, no more than 4 hours following collection; within 1 hour preferred (While being transported to processing lab from clinical site, keep sample on ice).

B. Whole Urine Sample (GenProbe PCA3 Assay):

The first 12.5 ml of urine will be prepped by placing 2.5mls of whole urine into each of 5 GenProbe Urine Specimen Transport Tubes for PCA3 (see below). If less than 12.5 ml is procured, then the entire specimen will be prepped in GenProbe Specimen Transport Tubes. The remainder of the voided urine will be prepped for urine sediment isolation (see below).

Instructions for processing whole urine aliquots for GenProbe:

- I. Invert initial voided urine sample (in urine collection cup) 5 times to re-suspend cells. Do not shake as it will cause foam and bubbles.
- II. Using the transfer pipette, transfer 2.5 ml of urine to an appropriately labeled Gen-Probe Urine Specimen Transport Tube (The correct volume of urine has been added to the transport tube when the fluid level is between the black fill lines).
- III. Screw on the transfer tube cap tightly, then invert the transport tube 5 times to mix.
- IV. Four (4) additional aliquots of processed urine specimens will be made by following the same procedures in steps B.I through B.III above, volume permitting. There should be a total of 5 processed urine specimens. Label the specimens with the provided Specimen Labels. All processed urine specimens should be frozen at -70°C or colder following processing & labeling.

C. Urine Pellet and Supernatant:

After the whole urine has been collected for the PCA3 assay as described above, the remainder will be used for urine sediment processing (If there is < 30 ml of urine after whole urine procurement then urinary sediment processing will not be possible). Urine samples should be placed on ice or at 4°C immediately and refrigerated for no longer than 4 hours if not processed sooner.

Depending on the remaining volume of urine after PCA3 is aliquoted, follow the following plan for creating urinary sediment:

- If there is 60-90 ml - divide sample into two equivalent samples, one for RNeasy prep and the second one for a PBS pellet
- If there is 30- 60 ml - create just one sample 30-40 ml for RNeasy sample (PBS pellet will not be created)
- If there is <30 ml - no urine sediment will be processed

Processing to Procure Urine Pellet and Supernatant:

- 1) Invert the urine collection cup 5 times to re-suspend the cells.
- 2) Transfer/divide remaining whole urine into two (sample 2A and 2B) 50 ml sterile conical tubes -2A will be used to generate the RNeasy pellet sample and 2B will be the PBS pellet sample.

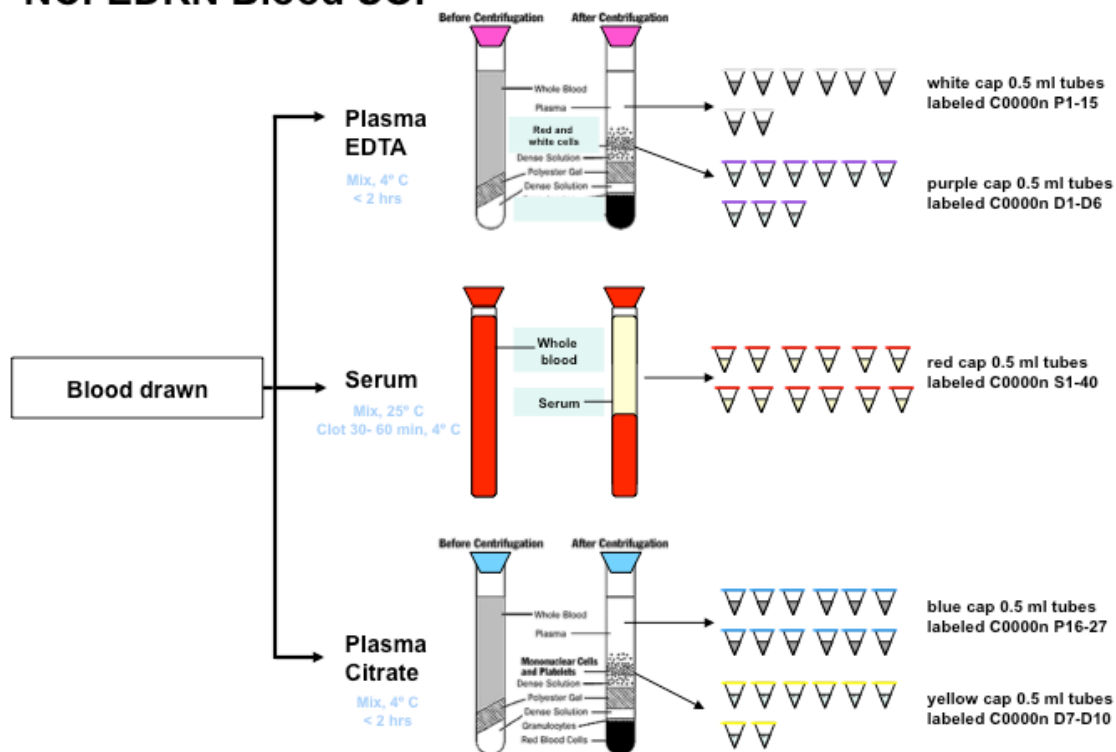
Steps 3-14 are done in parallel for samples 2A and 2B (RNeasy and PBS pellet)

- 3) Centrifuge: 10 min, $1000 \times g$ at 4°C . Do not use brakes.
- 4) Remove supernatant, taking care not to disturb the pellet.
 - a. To store supernatant for PCA3 trial: Decant the supernatant into 2 tubes as 5 ml and 2 tubes as 2 ml aliquots. Label with provided Specimen IDs.
- 5) Add 5 ml ice cold PBS (PBS must be free of calcium and Magnesium Chloride) to the remaining pellet.
- 6) Re-suspend pellet thoroughly by pipetting up and down with a sterile pipette.
- 7) Transfer suspension to a 15 ml conical tube.
- 8) Centrifuge: 10 min, $1000 \times g$ at 4°C . Do not use brakes.
- 9) Remove and discard this supernatant, taking care not to disturb the pellet.
- 10) Add 1 ml ice cold PBS.
- 11) Re-suspend pellet thoroughly by pipetting up and down with a sterile pipette.

- 12) Split the 1.0 ml PBS re-suspended pellet into two 1.7 ml Eppendorf tubes (500µl in each tube).
- 13) Centrifuge: 10 min, 700 x g at 4°C.
- 14) Decant taking care not to disturb pellet.
- 15) RNAlater Pellet (one tube for PCA3 trial; two tubes for non-PCA3 trial)
 - a. Re-suspend (vortex) pellet in 250µl of RNAlater.
 - b. Store at 4°C for 24-72 hours, then transfer the sediment tube to -70°C or colder in appropriate freezer box.
- 16) PBS Pellet (one tube for PCA3 trial; two tubes for non-PCA3 trial)
 - a. Store pellet in Eppendorf tube at -70°C in appropriate freezer box

NCI-EDRN Blood Processing Protocol

NCI-EDRN Blood SOP



Blood collection occurs at times the subject was receiving venopuncture for other routine tests, in order to avoid unnecessary blood draws.

Order of Blood Draw

1. Plasma Citrate-CPT Buffy Coat Specimens (BD Vacutainer Plasma Citrate tubes - Light blue cap tubes)
2. Serum (BD Vacutainer Serum tubes - Red cap tubes)
3. EDTA (BD Vacutainer Plasma EDTA tubes - Lavender cap tubes)

Order of Tube Processing

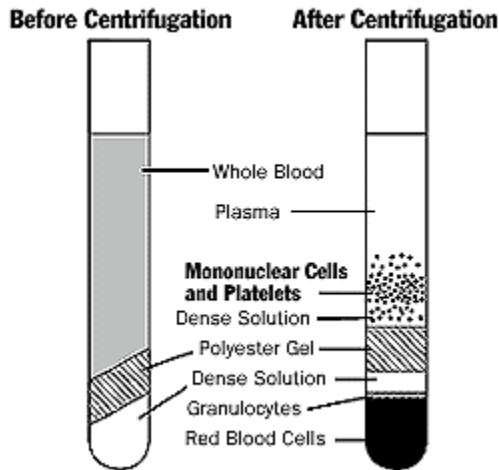
1. Plasma Citrate-CPT Buffy Coat Specimens
 - a. Keep light blue cap tubes at room temperature (25°C).
2. Serum Specimens
 - a. Keep red cap tubes at 4°C
3. Plasma and Cellular Fraction Specimens
 - a. Keep lavender cap tubes at 4°C

1. **Citrate-CPT Buffy Coat** (as described in

<http://www.bd.com/vacutainer/products/molecular/citrate/procedure.asp>)

- 1) Store the CPT tubes upright in a cardboard box at room temperature (25° C) until blood draw (Additive is light sensitive and if exposed to light could result in absence of the buffy coat).
- 2) Tubes must be filled to capacity until the vacuum is exhausted (The minimum volume of blood that can be processed without significantly affecting the recovery of mononuclear cells is approx 3.0 mL for 4.0 mL tube).
- 3) After the blood is drawn, invert the tube 8-10 times to mix anticoagulant additive with blood. Vigorous mixing can cause hemolysis.
- 4) Keep this tube at room temperature until centrifugation. Blood samples should be centrifuged within two hours of blood collection for best results.
- 5) Remix the sample immediately prior to centrifugation by gently inverting the tube 8-10 times.
- 6) Centrifuge the sample at room temperature (The principle of separation depends on the density gradient and the density of component varies with temperature).
 - a. Centrifuge for a minimum of 20 min at 1500-1800 RCF, g at room temperature.
 - i. Can centrifuge for 25 minutes at 2800 rpm (approximately 1528 g) at 25°C to achieve the same results.
 - ii. Centrifugation of the tube up to 30 min has the effect of reducing RBC contamination of the mononuclear cell layer.
- 7) Isolation of Plasma Citrate:
 - a. Remove top (plasma) layer and aliquot plasma in 100 ul into blue cap 0.5 ml tubes labeled C0000n P16-27 (for NCI).

- i. Aliquot any remaining volume into 200 ul into blue cap 0.5 ml tubes labeled C0000n (for lab) – label these tubes with numbers starting after last recorded white cap tube which should start at P28.
- b. Store aliquots at -80°C or colder until shipping to NCI.
 - i. NCI box labeled EDRN C0000n
 - ii. Lab box labeled EDRN



8) Isolation of Cellular Fraction from CPT Tube – conducted after removal of the plasma fraction:

(Mononuclear cells will be in a whitish layer under the plasma and above the gel; see picture above.)

- a. Remove the lymphocyte and monocyte band (see above picture) and transfer to a 15 ml conical centrifuge tube.
- b. Add PBS (pH=7.0) to attain 10 ml final volume.
- c. Invert the sample 5 times to remix the sample.
- d. Centrifuge at 300g (RCF) for 15 minutes at room temperature (25°C).
- e. Aspirate and discard supernatant without disturbing the pellet.
- f. Resuspend the pellet and then add 10 ml PBS.
- g. Mix the sample by inverting 5 times.
- h. Centrifuge at 300g (RCF) for 15 minutes at room temperature (25°C).
- i. Aspirate and discard supernatant without disturbing the pellet.
- j. Resuspend the pellet and then add 10 ml PBS.
- k. Mix the sample by inverting 5 times.
- l. Centrifuge at 300g (RCF) for 15 minutes at room temperature (25°C).
 - i. After this spin, decrease the temperature on the centrifuge to 4°C .
- m. Aspirate and discard the supernatant without disturbing the pellet.
- n. Resuspend the pellet in 500 ul PBS.

- o. Divide the cellular fraction into 100 ul aliquots in yellow cap 0.5 ml tubes labeled C0000n D7-D10 (for NCI).
 - i. Aliquot any remaining volume into 200 ul into yellow cap 0.5 ml tubes labeled C0000n (for lab) – label these tubes with numbers starting after last recorded purple cap tube which should start at D11.
- p. Store aliquots at -80°C or colder until shipping to NCI.
 - i. NCI box labeled EDRN C0000n
 - ii. Lab box labeled EDRN

2. Serum (processing consistent with

http://www.bd.com/vacutainer/pdfs/blood_collection_tubes_product_insert_VDP40035.pdf)

- 1) Store tubes at 4-25°C.
- 2) Tubes must be filled to capacity until vacuum is exhausted.
- 3) After blood is drawn, invert the tube 5 times to mix with the particles in the white film on the interior surface to activate clotting.
- 4) Allow blood to clot. Minimum clotting recommended time is 30 min (maximum time is 60 min) at room temperature (25°C) and then place tubes on ice.
- 5) Blood samples should be centrifuged within two hours of blood collection for best results. Centrifuge for a minimum of 10 min at 1000 - 1300 RCF, g at 4°C.
 - a. Can centrifuge for 15 minutes at 1500g (2500 rpm) at 4°C.
- 6) Remove 100 ul of serum (supernatant post-centrifugation) and place in red cap 0.5 ml tubes labeled C0000n S1-S40 (for NCI).
 - a. Aliquot any remaining volume into 200 ul into red cap 0.5 ml tubes labeled C0000n S41-Sn (for lab).
 - b. Hemolyzed serum samples are to be excluded. Normal serum is clear and straw colored whereas hemolyzed serum has a pink or red hue.
- 7) Store samples in -80°C or colder until shipping to NCI.
 - a. NCI box labeled EDRN C0000n
 - b. Lab box labeled EDRN

3. Plasma EDTA (processing consistent with

http://www.bd.com/vacutainer/pdfs/blood_collection_tubes_product_insert_VDP40035.pdf)

- 1) Store tubes at 4-25°C.
- 2) Tubes must be filled to capacity until vacuum is exhausted.
- 3) After blood is drawn, invert the tube 8-10 times to mix the additives with the blood.
- 4) Blood must be placed immediately on ice and should be centrifuged within two hours of blood collection for best results.

- 5) Centrifuge for a minimum of 10 min at ≤ 1300 RCF, g.
 - a. Can centrifuge for 15 minutes at 1500g (2500 rpm) at 4°C.
- 6) Plasma EDTA collection:
 - a. Remove 100 ul of the plasma (top layer) and aliquot into white cap 0.5 ml tubes labeled C0000n P1-P15 (for NCI).
 - i. Aliquot any remaining volume into 200 ul into white cap 0.5 ml tubes labeled C0000n P28-Pn (for lab).
 - b. Store at -80°C or colder until shipping to NCI.
 - i. NCI box labeled EDRN C0000n
 - ii. Lab box labeled EDRN
- 7) Cellular Component EDTA collection: The remaining cellular fraction after removal of EDTA-plasma includes red cells and white cells.
 - a. Gently vortex the tube for 10 seconds.
 - b. Make 6 aliquots of the admixed cellular fraction into 100 ul aliquots into purple cap 0.5 ml tubes labeled C0000n D1-D6 (for NCI).
 - i. Aliquot any remaining volume into 200 ul into purple cap 0.5 ml tubes labeled C0000n D11-Dn (for lab).
 - c. Store samples in -80°C or colder until shipping to NCI.
 - i. NCI box labeled EDRN C0000n
 - ii. Lab box labeled EDRN

DNA-RNA extraction

From each tumor focus, up to three cores were taken for DNA extraction and up to three cores for RNA extraction by 1.5mm biopsy punch. For RNA, tissue cores were homogenized in TRIzol (Invitrogen, Carlsbad, CA) and then isolated according to manufacturer's protocol. For DNA, the tissue cores were first digested at 55 °C for up to 16 hours in lysis buffer (TE, NaCl, SDS, Proteinase K and nuclease-free water). Next the lysate was treated with RNase A (Qiagen, Valencia, CA), and then DNA was isolated using phenol-chloroform. After isolation, both DNA and RNA were purified by ethanol precipitation method and suspended in nuclease-free water. The extracted RNA was treated with DNase I (Invitrogen) prior to quantification.

DNA quality assessment

To assess the integrity of genomic DNA, gel electrophoresis was performed with 50ng of DNA. Samples with little to no visible degradation were selected as candidates for DNA-sequencing and were sent to the Broad Institute of MIT and Harvard for genome-wide analysis on the Affymetrix Single-Nucleotide Polymorphism (SNP) Array 6.0 (Affymetrix, Santa Clara, CA) platform. For selection of samples for DNA-sequencing, parameters including tumor purity and average ploidy calculated from SNP array data were evaluated ¹.

RNA quality assessment

The quality of the RNA was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with Agilent RNA 6000 Nano Kit according to the manufacturer's instructions ². RNA Integrity Number (RIN) is a standardized and objective parameter to assess the quality of RNA. A score ranging from 10 to 1 is assigned for intact to completely degraded RNA respectively. For whole transcriptome sequencing, the average RIN for the samples used was 8.4.

Biobank Database

Pathological features included overall Gleason score, percentage of Gleason pattern 4, secondary tumor focus, identification of high-density and low-density tumors, percentage and localization of tumor, surgical margin status, and extraprostatic extension. Special features of the tumor that could be informative in distinguishing and molecularly classifying tumors in the future such as mucinous changes, ductal features, etc. were also documented. FISH and RT-PCR were used to validate various gene rearrangements occurring in prostate cancer, and *ERG* rearrangement status in particular was examined in all cases and recorded in the database. When frozen blocks were cored for nucleic acid extraction, corresponding RIN values were determined and recorded for quality verification.

Sequencing Data

RNA samples were sequenced using Illumina Genome Analyzer II at WCMC and researchers were given access to expression profiling data. The Broad Institute sequenced DNA samples provided by WCMC and the genetic data generated was submitted to the database of Genotypes and Phenotypes (dbGaP), a NCBI data distribution service (<http://www.ncbi.nlm.nih.gov/gap>), and the Sequence Read Archive (SRA), serviced by the U.S. National Library of Medicine (<http://www.ncbi.nlm.nih.gov/sra>). In addition to genotype, dbGaP also requires study documents, de-identified phenotype information, sufficient metadata, and assurance of appropriate informed consent. This data is divided into open-access data for public and controlled-access data requiring authorization. SRA stores short read data and associated metadata that can be quickly accessed by researchers.

References

1. Berger MF, Lawrence MS, Demichelis F, *et al.* The genomic complexity of primary human prostate cancer. *Nature* 2011; 470(7333):214-20.
2. Schroeder A, Mueller O, Stocker S, *et al.* The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol* 2006; 7:3.