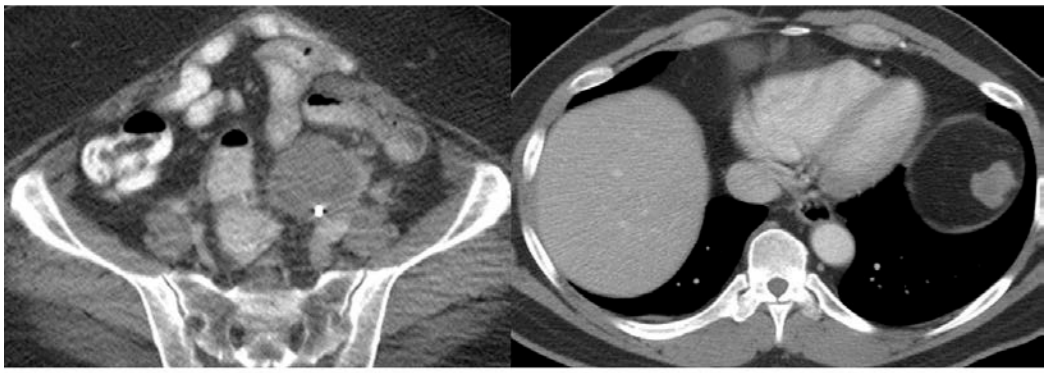
DECAMP

BIOMARKER RESEARCH

Detection of Early Lung Cancer among Military Personnel

ACRIN 4703 / ACRIN 4704
Biospecimen Procedure Manual



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I. INTRODUCTION

The ACRIN 4703 / ACRIN 4704 Biospecimen Procedure Manual provides instructions for collecting, processing, storing, shipping, and documenting all biospecimens for both DECAMP studies: **ACRIN 4703**, *Detection of Early lung Cancer Among Military Personnel Study 1 (DECAMP-1): Diagnosis and Surveillance of Indeterminate Pulmonary Nodules* and **ACRIN 4704**, *Detection of Early Lung Cancer Among Military Personnel Study 2 (DECAMP-2): Screening of Patients with Early Stage Lung Cancer or at High Risk for Developing Lung Cancer*.

To successfully meet the study objectives, it is critical that the participating sites follow the instructions and guidelines outlined in this manual.

Quality Control (QC) review of the biospecimen collection processes will occur at the Boston University Biospecimen Core (Dan Remick, MD) and the Pathology Core at M. D. Anderson Cancer Center (Ignacio Wistuba, MD). QC review of study images will be performed by the ACR Imaging Core Laboratory. These reviews will be performed in a timely fashion as part of ACRIN standard operating procedures. If any protocol deviations or technical issues are identified during the review, the QC Specialist at each laboratory will contact the site to provide feedback expeditiously. This will allow the site to make the necessary adjustments early in the conduct of the study.

The DECAMP Research Team wishes to thank you in advance for your diligence in adhering to the procedures described in this manual to ensure the integrity of the image data collected for the study. Please do not hesitate to contact the DECAMP Research Team or the Principal Investigators if you have any questions.

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II. SPECIMEN PROCUREMENT PROCESS

A. Standard Safety Procedures for All Biospecimen Collection

In the collection and handling of biospecimens in this study, universal precautions, Occupational Safety and Health Administration guidelines (OSHA) and institutional requirements should be followed. Universal precautions are a method of infection control in which all human tissue, blood and body fluids are treated as if they are infectious. Be sure to wear appropriate personal protective equipment (e.g., gloves, yellow gown, eye protection, etc).

All materials (e.g., tubes, needles, pipettes, etc) must be properly disposed of in biohazard containers in accordance with institutional requirements. All equipment used in the collection, storage and shipping must be labeled as biohazard.

B. Confidentiality

All efforts will be made to maintain the confidentiality of the studies' participants. All information collected will be protected per ACRIN and DoD policies and procedures along with federal regulatory guidelines. Access to study data will be limited to the ACRIN - DECAMP staff. All data will be maintained in a manner consistent with Title 21 Code of Federal Regulations (CFR) Part 11. In addition, access to the data management system will be limited to designated staff through use of confidential, individualized login ID and password.

C. Data Retention

The data from these studies will be maintained following completion of the studies or until no longer required for research. Data will be destroyed as required by the ACRIN Record Retention Policy, DoD regulations and federal regulatory guidelines.

D. Informed Consent

Human research subjects are protected by informed consent procedures in accordance with Title 45 CFR Part 46 and Title 21 CFR Part 50. All biospecimen samples will be collected from participants who have agreed to participate in the study and have signed the local site-IRB-approved DECAMP-1 or DECAMP-2 Informed Consent Form.

All biospecimens received by Boston Medical Center lab must have participant consent for biospecimen collection and tissue banking. The individual participant Informed Consent Forms are maintained and stored by the enrolling institution and there is no requirement for the Boston Medical Center lab to receive these forms. However, confirmation of consent will be recorded by the enrolling institution on the biospecimen transmittal form which accompanies the biospecimens shipped to the Boston Medical Center lab.

The DECAMP-1 and DECAMP-2 Informed Consent Forms grant permission for study investigators to request and obtain surgical material (such as pathologic tissues), blood and derivatives, urine, and sputum, and to use those samples for research involving molecular studies on the development of lung cancer and/or for other diseases.

E. HIPAA

ACRIN - DECAMP enrolling institutions will administer a local site-IRB-approved HIPAA authorization or alternative notification describing use and disclosure of protected health information to DECAMP participants at the time of administration of the Informed Consent Form in accordance with their local IRB / institutional guidelines.

The Boston Medical Center lab database, which is primarily an inventory and tracking system, does not contain specific participant health identifiers for study participants. The enrolling institution will identify the participant by the study and case ID #; all biospecimens will be de-identified of participant health identifiers using a barcode system, described in this manual, by the enrolling institution prior to shipping to the Boston Medical Center lab. Once the Boston Medical Center lab receives the biospecimens, the lab will use the barcode system to log the biospecimens and to identify the participant for the ACRIN 4703 DECAMP-1 or ACRIN 4704 DECAMP-2 study.

If any information is conflicting or missing from the specimen transmittal form or the biospecimens, the Boston Medical Center lab will contact the enrolling institution for the missing information or clarification before the specimens can be entered into the Boston Medical Center lab database and stored.

III. SPECIMEN COLLECTION KIT, LABELING AND COLLECTION SUPPLIES

A. Kit Contents and Design

1. Kit Contents:

The standard kit is for the collection, processing, storage, and shipping of whole blood, serum, plasma, urine, sputum, buccal, nasal, and bronchial brushings, and pathology tissue as specified by the study protocol.

Standard kit contents:

- 2 lavender top venous blood collection tubes containing EDTA for plasma (5-7 mL of blood per tube);
- 1 red top venous blood collection tubes containing no additive, silicone coated for serum (5-7 mL of blood per tube);
- 1 Streck cell-free DNA BCT tubes (10mL of blood per tube) for circulating DNA as detailed below;
- 2 PAXgene tubes (2.5 mL of blood per tube) for RNA as detailed below;
- 2 mL cryovials x 24
- 5 mL cryovials x 6;
- Urine collection container (with towelette)
- Sputum collection container x 2;
- Patient Sputum Collection Instructions
- Postage paid corrugated box addressed to Boston University Laboratory (for sputum specimen)
- Nasal brushes x 2;
- Nasal speculum;
- Bronchial brushes x 4;
- Buccal / mouth scraper x 5;
- Formalin containers x 5;
- Biospecimen Transmittal Form;
- Bronchical Biopsy Formalin Fixed Biospecimen Transmittal Form
- Surgical Lung Fresh Frozen Biospecimen Transmittal Form;
- Surgical Lung Formalin Fixed Biospecimen Transmittal Form

Sites that collect and process the 'optional' biospecimens (buffy coat, mononuclear cells, urine metabolomics, nasal brush for single cell sorting) will receive addional supplies. Detailed instructions for collecting and processing the optional biospecimens are included in Appendix III.
Please notify Boston University by e-mail (jackcunn@bu.edu) if your site is collecting one or more of the optional specimens and requires additional supplies.

Optional kit contents:

- 2 yellow top venous blood collection tubes containing ACD Solution A of trisodium citrate, 22.0 g / L; citric acid, 8.0 g / L; and dextrose 24.5 g / L, 1.5 mL (5-7 mL of blood per tube);
- Urine collection container for metabolic study with towelette;
- 2 mL cryovials x 8
- 5 mL cryovials x 11
- Nasal brush x 1

Kits for the DECAMP 2 annual time point 2 will not include the contents required for the bronchial brushings and biopsies.

2. Kit Design

All specimens collected in the ACRIN-DECAMP 4703 and 4704 studies and banked at the Boston Medical Center will be collected using the materials and protocols provided by Boston University. Each kit provides all materials necessary to collect, process, and ship biospecimens to the Boston Medical Center lab. All sample containers are labeled with a barcode, with a matching barcode label on the accompanying kit transmittal form; each kit is designed to meet the collection needs of the trial.

B. Barcode Labels

All specimen collection and storage containers within a kit will be labeled with a unique barcode sequence. They are generated using LABELVIEW Software. Baseline specimen barcodes are produced using a custom label design, consisting of a barcode corresponding to a 9-digit number. Specimens collected post-cancer diagnosis or at the DECAMP 2 annual follow-up time points have a 10-digit number. The specimen tracking system requires a unique barcode number for each specimen, so numbers are printed consecutively, and never repeated. Specimen barcodes are printed on a BRADY i5100 printer. All labels and ribbons are purchased from BRADY specifically designed for cryogenically stored samples, both for –80ºC and liquid nitrogen. Each barcode is printed in duplicate side-by-side or in sequence to provide one label for the specimen, and a matching label for the specimen transmittal form.

- DECAMP 1 & 2 Baseline – 9-digit label format: (xxxx-xxx-xx)
- DECAMP 1 & 2 Post Cancer Diagnosis – 10-digit label format (xxxx-xxx-X-xx).
- DECAMP 2 Year 1 Follow-up – 10-digit label format (xxxx-xxx-2-xx) (2nd specimen collection time point)
- DECAMP 2 Year 2 Follow-up – 10-digit label format (xxxx-xxx-3-xx) (3rd specimen collection time point)

C. Kit Distribution and Collection Supplies

Study sites will receive specimen collection kits, pre-labeled with a unique barcode specific for that kit and its contents. Sites also will receive one styrofoam frozen specimen shipper. These are to be used for return mailing of completed specimens. All sites will receive in bulk additional specimen collection supplies that will be used in the procurement, processing, and storage of all the biospecimens. Requests for kits and other collection supplies from enrolling centers should be directed to Boston University by e-mail (jackcunn@bu.edu). If requesting kits, include the next set of kit sequence numbers, a timeline or 'needed by' date, and your ACRIN site number. Kits are routinely processed in sets of 5, but additional kits can be assembled if needed and requested.

Once the requesting center has contacted Boston University, the materials will be sent as quickly as possible. The goal is to process requests within 2-3 business days.

Upon receipt of all kits and collection supplies, the study site should inspect the contents and confirm receipt or report missing or broken items to Jack Cunningham, Clinical Biospecimen Coordinator via e-mail (jackcunn@bu.edu).

IV. BLOOD COLLECTION PROCESS

A. Blood Collection Tubes

Approximately 50 mLs of blood will be collected during each blood collection visit. The technician must be familiar with the arrangement of blood collection tubes, the order in which the tubes are to be filled, the type of anticoagulant in each tube, and possible sources of error in handling each tube. These tubes are organized in the test tube rack in the following sequence:

- **RED TOP**
- **LAVENDER**
- **STRECK**
- **PAXGENE**

Blood will be collected in 6 vacutainer tubes:

- 2 lavender top venous blood collection tubes containing EDTA for plasma (5-7 mLs of blood per tube);
- 1 red top venous blood collection tubes containing no additive, silicone coated for serum (5-7 mL of blood per tube);
- 1 Streck Cell-Free DNA BCT tube (10 mL of blood per tube) containing preservative reagent for circulating DNA as detailed below;
- 2 PAXgene tubes (2.5 mL of blood per tube) for RNA as detailed below;

B. Blood Plasma Preparation

1. Materials and Equipment

- Human blood sample;
- Vacutainer tubes with lavender top x 2;
- Serological pipettes of appropriate volumes (sterile);
- 15 mL Centrifuge tubes x 2;
- 2 mL Cryovials x 8;
- Benchtop centrifuge with swing-out rotor and appropriate carriers;
- –80ºC freezer;
- Wet ice in bucket.

2. Procedure

1. ***Plasma samples should be processed and stored within two hours of blood collection.***
2. Draw blood into two (2) lavender vacutainer tubes. Be sure to draw the full volume to ensure the correct blood-to-anticoagulant ratio.
3. After collection in vacutainer, gently mix the blood by inverting the tube 10 times to mix blood and anticoagulant and place mixed tube in wet ice.
4. Centrifuge lavender top tubes for 15 minutes at 1000-2000 RCF (generally 1300 RCF) in a refrigerated +4˚C centrifuge. *Please refer to speeds and times recommended by manufacturer.* Do not use brake to stop centrifuge.
5. Post centrifugation, three layers will develop: (from top to bottom) plasma, leukocytes (buffy coat), and erythrocytes (red blood cells, RBCs).
6. Carefully collect the plasma layer at room temperature from both lavender top vacutainers and pool into a 15 mL centrifuge tube. Take care not to disrupt the leukocytes (buffy coat) layer or transfer any cells. **DO NOT DISCARD LAVENDAR TOP TUBE – IF YOU CHOOSE TO DO BUFFY COAT EXTRACTION – YOU WILL NEED TO KEEP THE REMAINDER FOR THE BUFFY COAT SOP (refer to Appendix IV for instructions).**
7. Spin in a refrigerated +4˚C centrifuge at 1500g for 5 minutes to remove all potentially remaining cells.
8. Inspect plasma for turbidity. Turbid samples should be centrifuged and collected again to remove remaining insoluble matter. Pipet the plasma into appropriate sized aliquots, attempting not to disturb any of the cell pellet. Transfer plasma into a total of 8 cryovials: 4 cryovials (250µL/vial), 2

cryovials (500µL/vial) and remaining plasma into 2 cryovials (1mL/vial). All the samples are labeled appropriately. At the end of the procedure check that all aliquot vial caps are secure.

9. After aliquoting the required vials, pipet and save any remaining plasma into an extra cryovial. Use a cryovial that is labeled with a barcode.
10. Store vials upright in a labeled specimen box in a –80ºC freezer. All specimens should remain at –80ºC prior to distribution.
11. Document the data point information appropriately on the Case Report Forms:
    - Date and time of blood collection;
    - Number and volume of aliquots prepared;
    - Date and time into –80ºC;
    - Any freeze-thaw that occurs with a sample for any reason;
    - Additional information as specified in the Case Report Forms.

C. Serum Preparation

1. **Materials and Equipment**
    - Human blood sample;
    - Vacutainer tubes with red top x 1 (containing either no additive or a clot activator);
    - Serological pipettes of appropriate volumes (sterile);
    - Centrifuge tubes;
    - 2 mL Cryovials x 3;
    - Benchtop centrifuge with swing-out rotor and appropriate carriers;
    - –80ºC freezer.

2. **Procedure**

1. ***Serum samples should be processed and stored within two hours of blood collection.***

2. For serum collection, draw whole blood into red top vacutainer tube containing no anticoagulant.
3. Incubate in an upright position at room temperature for 30-45 min (no longer than 60 min) to allow clotting. If using a clot-activator tube, invert carefully 5-6 times to mix clot activator and blood before incubation. While each successive tube is filling, turn the filled tube upside-down and return it to the upright position. This is one complete inversion. For optimal results, invert the tubes 5 more times.
4. After blood has clotted, centrifuge in a refrigerated +4˚C centrifuge for 15 minutes at manufacturer's recommended speed (usually 1000-2000 RCF). Do not use brake to stop centrifuge.
5. After centrifugation, use a disposable transfer pipet to draw the serum (top layer) off into a 15 mL polypropylene collection tube.
6. Aliquot volume is 250 µL of serum transferred into 2 cryovials and 1 mL of serum into the remaining 1 cryovial for a total of 3 tubes. At the end of the procedure, check that all aliquot vial caps are secure.
7. Store vials upright in a labeled specimen box in an –80ºC freezer. All specimens should remain at –80ºC prior to samples collection for delivery to the appropriate study core lab.
8. After aliquoting the required vials, pippet and save any remaining into an extra cryovial. Use a cryovial that is labeled with a barcode.
9. Document the data point information appropriately on the Case Report Forms:
    - Date and time of blood collection;
    - Number and volume of aliquots prepared;
    - Date and time into –80ºC;
    - Any freeze-thaw that occurs with a sample for any reason;
    - Additional information as specified in the Case Report Forms.

D. Streck Cell-Free DNA Tube Preparation

1. Materials and Equipment

- Human blood sample;
- Streck Cell-Free DNA BCT Tube x 1;
- Serological pipettes of appropriate volumes (sterile);
- 15 mL centrifugation tube x 1;
- 2 mL Cryovial x 4
- Benchtop centrifuge with swing-out rotor and appropriate carriers;
- –80ºC freezer

2. Procedure

1. Using a blood collection set and a holder, collect 10 mL blood into the Streck Cell-Free DNA BCT® Tube using your institution's recommended procedure for standard venipuncture technique.
2. Use the following techniques to prevent possible backflow:
   - Place donor's arm in a downward position.
   - Hold tube with the stopper in the uppermost position so that the tube contents do not touch the stopper or the end of the needle during sample collection.
   - Release tourniquet as soon as blood starts to flow into tube or within 2 minutes of application.
3. Immediately after blood collection, gently invert the Streck Cell-Free DNA BCT® Tube 8 to 10 times.
4. Store the Streck Cell-Free DNA BCT® Tube upright at 6˚C to 37˚C for a maximum of 14 days before processing.
5. When ready to process, centrifuge samples at 1600 x g for 10min at <u>room temperature</u> (*first centrifugation)*. <u>Do not use brake to stop centrifuge.</u>
6. Carefully transfer the upper plasma layer to a new 15 mL centrifugation tube by pipette tip.
7. Centrifuge samples at 1600 x g for 10min at <u>room temperature</u> (*second centrifugation*). <u>Do not use brake to stop centrifuge.</u>
10. Carefully transfer the upper plasma into cryovials. Aliquot at least 1 mL plasma per vial. Depending on the total volume of plasma isolated, fill 2-4 cryovials. At the end of the procedure, check that all aliquot vial caps are secure. Store labeled tubes at -80˚C.
8. Document the data point information appropriately on the Case Report Forms:
   - Date and time of blood collection;
   - Number and volume of aliquots prepared;
   - Date and time into storage upon receipt, into ambient temperature following blood collection, into –80ºC freezer;
   - Any freeze-thaw that occurs with a sample for any reason;
   - Additional information as specified in the Case Report Forms.

E. PAXgene Preparation

PAXgene™ Blood RNA Tube <u>should be drawn last</u> in the phlebotomy procedure, so the interior volume of the blood collection set used during phlebotomy can be primed.

1. Materials and Equipment

- PAXgene Blood RNA Tubes
- –20ºC freezer
- –80ºC freezer

2. Storage Conditions for Materials

PAXgene RNA spin columns, PAXgene Shredder spin columns, proteinase K, and Buffers BR1 to BR5 can be stored dry at room temperature (15–25°C).

The RNase-Free DNase Set, which contains DNase I, Buffer RDD, and RNase-free water (tube), is shipped at ambient temperature. Store at 2–8°C all components of the RNase-Free DNase Set immediately upon receipt.

3. Procedure

Ensure that the PAXgene™ Blood RNA Tube is at 18˚C to 25˚C prior to use and properly labeled with participant identification.

1. Using a blood collection set and a holder, collect blood into the PAXgene™ Blood RNA Tube using your institution's recommended procedure for standard venipuncture technique.
2. Use the following techniques to prevent possible backflow:
   - Place donor's arm in a downward position.
   - Hold tube in a vertical position, below the donor's arm during blood collection.
   - Release tourniquet as soon as blood starts to flow into tube.
   - Make sure tube additives do not touch stopper or end of the needle during venipuncture.
3. Allow at least 10 seconds for a complete blood draw to take place. Ensure that the blood has stopped flowing into the tube before removing the tube from the holder. The PAXgene™ Blood RNA Tube vacuum is designed to draw 2.5 mL of blood into the tube.
4. Immediately after blood collection, gently invert the PAXgene™ Blood RNA Tube 8 to 10 times.
5. Store the PAXgene™ Blood RNA Tube upright at room temperature (18˚C to 25˚C) for a minimum of 2 hours before transferring to a –20˚C freezer for 24 hours. Transfer to a -20 is highly preferred but not necessary.
6. After 24 hours, transfer the PAXgene tubes to a –80ºC freezer
7. Stand the PAXgene™ Blood RNA Tube upright in a wire rack. Do not freeze tubes upright in a styrofoam tray as this may cause the tubes to crack.
8. Document the data point information appropriately on the Case Report Forms:
   - Date and time of blood collection;
   - Number and volume of aliquots prepared;
   - Date and time into storage upon receipt, into ambient temperature before blood collection, into –20ºC freezer and subsequently into –80ºC freezer;
   - Any freeze-thaw that occurs with a sample for any reason;
   - Additional information as specified in the Case Report Forms.

Please select the hyperlink for PAXgene Blood RNA Collection
Or type URL http://www.preanalytix.com/videos/rna-tube-collection-video/

Please select the hyperlink for PAXgene Blood RNA Freezing
Or type URL http://www.preanalytix.com/videos/rna-tube-freezing-video/

V. URINE COLLECTION

Urine Collection (Midstream Clean Catch)

Midstream Clean Catch Specimen is the preferred type of specimen for culture and sensitivity testing to reduce incidence of cellular and microbial contamination. Before a patient voids, please make sure the patient is well hydrated and has a full bladder for an optimized urine volume collection.

1. Materials and Equipment

- Sterile urine collection container; with soap towelette
- 5 mL cryovials x 6;
- –80ºC freezer.

2. Procedure

1. Instruct participants to first clean the urethral area with a castile soap towelette.
2. Instruct participants to collect urine midstream into a clean approved container. Excess urine should be voided into the toilet. This method of collection should be conducted in the clinic.
3. Seal the container immediately and place on ice or in the refrigerator (2˚C to 4˚C).
4. Aliquot 5 mL of the urine into each of the six cryovials provided.
5. Urine container and any excess urine should be disposed of properly.
6. At the end of the procedure, check that all aliquot vial caps are secure and that all vials are labeled appropriately.
7. Store vials upright in a labeled specimen box in an –80ºC freezer. All specimens should remain at –80ºC prior to distribution.
8. Document the data point information appropriately on the Case Report Forms:
   - Date and time of urine collection;
   - Number and volume of aliquots prepared;
   - Date and time into –80ºC;
   - Any freeze-thaw that occurs with a sample for any reason;
   - Additional information as specified in the Case Report Forms.

VI. BUCCAL EPITHELIUM COLLECTION

1. Materials and Equipment

- 5 mouth scrapers;
- 2 mL cryovial x 1;
- RNAprotect Cell Reagent (Qiagen #76526) ;
- RNaseZap (Ambion 9780; 9782);
- –80ºC freezer.

2. Procedure

1. Place the tube filled with 1.5 mL of RNAprotect, labeled with the solution name and collection date in a tube rack.
2. Treat the sterilized wire cutter with a few sprays of RNase Zap and wipe with paper towels, then wipe with 2 alcohol wipes.
3. Ask the participant to rinse his/her mouth with water to remove any debris.
4. Scrape the inside left cheek with 5 to 10 vigorous strokes, being careful not to break the skin.
5. Put used scraper into the 2 mL cryovial . Plunge up and down to wash off cells into the tube. Discard the scraper.
6. Repeat steps 4 and 5 with four more scrapers, detaching the head of the 5th scraper and leaving it in the cryovial. You may want to use a sterile clamp to help ease breaking off scraper head into the cryovial.
7. At the end of the procedure, check that the cryovial cap is secure and labeled appropriately.
8. Store the cryovial upright in a labeled specimen box in a –80ºC freezer. All specimens should remain at –80ºC prior to distribution.
9. Document the data point information appropriately on the Case Report Forms:
   - Date and time of buccal epithelium collection;
   - Number and volume of the aliquot prepared;
   - Date and time into –80ºC;
   - Any freeze-thaw that occurs with a sample for any reason;
   - Additional information as specified in the Case Report Forms.

VII. NASAL EPITHELIUM COLLECTION

1. Materials and Equipment

- Cytopak soft brushes;
- 2 mL cryovial x 2;
- RNAprotect Cell Reagent (Qiagen #76526);
- RNaseZap (Ambion 9780; 9782);
- 1% Lidocaine (either syringe with removable needle or spray bottle for administration);
- Wire cutter;
- 70% isopropyl alcohol wipes;
- Nasal speculum;
- –80ºC freezer.

2. Procedure

1. Place the tube filled with 1.5 mL of RNAprotect, labeled with the solution name and collection date, in a tube rack.
2. Treat the wire cutter with a few sprays of RNase Zap and wipe with paper towels, then wipe with 2 alcohol wipes.
3. Ask participant to blow his/her nose to remove any debris.
4. OPTIONAL: Ask the participant if he/she would like you to use lidocaine to numb the nostril prior to collection. Please note procedure below in “Optional lidocaine” box.
5. Using a speculum to widen the left nostril, locate the inferior turbinate. A pen light may be useful.
6. With speculum still widening nostril, insert the brush into the nostril just past the inferior turbinate and press the brush against the nostril towards the outside of the nose, opposite the septum, while rotating the brush. This should take approximately 3 seconds.
7. Remove the brush from the nostril and place the brush head immediately in the tube with RNAprotect Cell Reagent.
8. Cut off the brush from the shaft with wire cutters.
9. Repeat steps 5 through 7 with the second brush (in the same nostril).
10. **An optional third brush for single cell analysis may be taken at select DECAMP sites: Walter Reed, Boston University Medical Center and University of California Los Angeles, see details in Appendix III.
11. At the end of the procedure, check that all aliquot vial caps are secure and labeled appropriately.
12. Vortex samples to ensure that all cells come into contact with RNAprotect Cell Reagent.
13. Store vials upright in a labeled specimen box in a –80ºC freezer. All specimens should remain at –80ºC.
14. Document the data point information appropriately on the Case Report Forms:
    - Date and time of blood collection;
    - Number and volume of aliquots prepared;
    - Date and time into –80ºC;
    - Any freeze-thaw that occurs with a sample for any reason;
    - Additional information as specified in the Case Report Forms.

Optional lidocaine:

Sites may offer to numb the participants’ nostril prior to biospecimen collection using the lidocaine administration procedure below:

- Load 1 mL of lidocaine into syringe (remove needle) or spray bottle.
- Have participant hold a paper towel under his/her nose.
- Have participant verbally and rapidly repeat the letter “K” over and over again. (Please explain that this helps prevent the lidocaine from getting into the throat, which can feel/taste unpleasant.)
- While participant is saying the letter “K”, quickly administer the lidocaine into the nostril against the outside of the nose, which is opposite the septum.

VIII. BRONCHIAL AIRWAY BRUSHINGS COLLECTION

1. Materials and Equipment

- Cellebrity endoscopic cytology brushes (Boston Scientific #1601) x 4;
- RNaseZap (Ambion 9780; 9782);
- RNAprotect Cell Reagent (Qiagen #76526);
- PBS;
- Wire cutter;
- 70% isopropyl alcohol wipes;
- –80ºC freezer.

2. Procedure

Tube	2 mL Cryovials containing:
A	1 mL of RNA protect Cell Reagent
B	1 mL of 1X PBS solution for proteomic analysis
C	1 mL of 1X PBS solution for DNA extraction
D *optional*	1 mL of RNA protect Cell Reagent (optional)

1. Label the 2 mL cryovials with appropriate solution names and collection date and place them in a bucket of ice.
2. Treat the wire cutter with a few sprays of RNase Zap and wipe with paper towels, then wipe with 2 alcohol wipes.
3. Perform endobronchial brushings before performing the endobronchial biopsies in the following section.
4. Proceed to the right mainstem bronchus. Obtain the endobronchial samples at the level of the RIGHT UPPER LOBE (RUL) takeoff. Obtain each brush at a site adjacent to the previous brushing within the right mainstem bronchus.
5. Take four separate brushings from this area. Using one brush per cryovial. If this area is grossly involved with a disease process (like tumor), take the brushings from the contralateral side.
6. Rub each cytology brush against the mucosa for 10 to 20 vigorous back and forth strokes. Rotate the brush along its long axis to obtain cells from the 360 degree surface of the brush. A visible abrasion should be at the site afterwards, which ensures good contact between the brush and mucosa. *Please try to minimize bleeding because it compromises the quality of the analysis.*
7. Retract the brush into the sheath and remove it from the bronchoscope.
8. Post-brushing, bring the brush out of the plastic sheath. Using the treated wire cutters, cut the brush where the plastic sheath ends and place into the cryovial containing the appropriate reagents.
9. Shake each tube vigorously.
10. Store Tube A, Tube B, and optional Tube D at –80°C.
11. Perform additional processing for Tube C:
    a. Vortex Tube C with the brush tip for about 10 seconds.
    b. Centrifuge Tube C with the brush inside the tube at 1500g for 10 minutes.
    c. Leaving the brush inside the tube and without disturbing cells, carefully remove the saline from Tube C. A small amount of saline (<20 µL) of saline may be retained inside the tube. This is very important to keep the cells in low volume without losing the cells. Pellet may not be visible.
    d. Store in –80ºC.
12. Document the data point information appropriately on the Case Report Forms:
    - Date and time of bronchial airway brushings collection;
    - Number and volume of aliquots prepared;
    - Date and time into –80ºC;
    - Any freeze-thaw that occurs with a sample for any reason;
    - Additional information as specified in the Case Report Forms.

IX. BRONCHIAL BIOPSY COLLECTION

Two bronchial samples (one formalin-fixed and one fresh-frozen) will be obtained for each predetermined site. Tissue sites will include:

(1) Right upper lobe (RUL) carina;
(2) Right middle lobe (RML) carina;
(3) Left upper lobe (LUL) carina;
(4) If autofluorescence bronchoscopy is performed, any abnormal sites should be biopsied for research purposes (in addition to any biopsies taken for clinical purposes).

1. Materials and Equipment

- Cryovials;
- Biopsy containers with 10% buffered formalin;
- Aluminum foils pieces (2x2 cm);
- Fine disposable forceps;
- Liquid nitrogen;
- Dry ice;
- –80ºC freezer.

2. Procedure

1. Record fluorescence ratio at the biopsy site.
2. Record location of biopsy (e.g., RUL , RML, LUL, etc).
3. Use clean (disposable) forceps. Clean forceps with 100% alcohol each time you reuse it.
4. For each site, take 2 bronchial biopsies of approximately 1.5 to 1.8 mm diameter from 2 adjacent sites, with 1 sample formalin-fixed and 1 sample fresh-frozen. As biopsy histology is critical, try not to crush the sample.
5. Label bronchial biopsy samples appropriately.
6. Fresh-frozen biopsies:
   a. Carefully transfer the biopsy specimen using forceps into the sterile aluminum foil piece, and wrap it up carefully.
   b. Put the aluminum-foiled specimen in a labeled cryotube.
   c. Flash freeze the tissue by putting the cryotube in the liquid nitrogen cooler for at least 1 minute. Alternate flash freezing procedure: 100% isopropyl (or methanol) with dry ice bath to snap freeze the tissue if liquid nitrogen is not available.
   d. Keep the cryotube in a –80˚C freezer until shipping.
7. Formalin-fixed biopsies:
   a. Carefully transfer the biopsy specimen using forceps into a labeled biopsy container with 10% buffered formalin.
   b. Process and embed the specimen in paraffin (within 48 [maximum] hours) and process into 4-5 slides. Clearly label the paraffin blocks, as well as each corresponding slide with the proper DECAMP patient ID.
   c. Ship 4-5 paraffin slides in a plastic slide box, inside a shipping container filled with dry ice to Boston University core lab facility with the other samples.
      - Prior to shipping, slides should be stored in a –80˚C freezer but can be kept in a –20˚C freezer for short-term storage.
8. Document the data point information appropriately on the Case Report Forms:
   - Date and time of bronchial biopsy collection;
   - Number and volume of aliquots prepared;
   - Date and time into –80ºC;
   - Any freeze-thaw that occurs with a sample for any reason;
   - Additional information as specified in the Case Report Forms.

X. SURGICAL LUNG SPECIMENS COLLECTION

If participant is scheduled for surgery, these procedures should be followed to store formalin-fixed and fresh-frozen surgical tumor and adjacent normal tissues.

1. Materials and Equipment

- 2 mL cryovials;
- 2 biopsy containers with 10% buffered formalin;
- Aluminum foil pieces (2 cm x 2 cm);
- Fine disposable forceps;
- Liquid nitrogen;
- –80ºC freezer.

2. Procedures

1. The intact operative specimen is sent as soon as possible to Surgical Pathology—fresh specimen for tumor (T) and adjacent normal lung (N) tissue collection.
2. Operating Room will page the Tissue Acquisition Core personnel to request pick up of the lung specimen from Surgical Pathology.
3. The pathologist, pathology assistant or resident/fellow will examine the specimen and, allowing for adequate tissue for histological diagnosis and assessment of margins, will remove a portion (0.5 cm x 0.5 cm x 0.5 cm) of the T and N tissue (0.5 cm x 0.5 cm x 0.5 cm).
4. Tissue collection assistant will be present in Surgical Pathology to snap freeze and fix the T as soon as possible to maximize the RNA preservation. The approximate time that elapses before freezing and fixation will be noted.
5. Two types of T and N tissue will be obtained: fresh frozen and formalin-fixed.
6. Fresh-frozen tissues:
   a. Individual tissue specimens should measure approximately 0.5 cm x 0.5 cm x 0.5 cm.
   b. Carefully transfer the T and N specimens into the aluminum foil piece, and wrap it up carefully.
   c. Put the aluminum-foiled specimen in a labeled 2 mL cryotube.
   d. Flash freeze the tissue by putting the cryotube in the liquid nitrogen cooler for at least 1 minute. Alternate flash freezing procedure: 100% isopropyl (or methanol) with dry ice bath to snap freeze the tissue if liquid nitrogen is not available.
   e. Keep the cryovial in a –80˚ C freezer until shipping.
7. Formalin-fixed biopsies:
   a. Individual tissue specimens should measure approximately 0.5 cm x 0.5 cm x 0.5 cm.
   b. Carefully transfer the tissue specimen into a labeled biopsy container with 10% buffered formalin.
   c. Process and embed the specimen in paraffin (within 48 [maximum] hours) and process into 10 or more slides. Clearly label the paraffin blocks, as well as each corresponding slide with the proper DECAMP patient ID.
   d. Ship 10+ paraffin slides in a plastic slide box, inside a shipping container filled with dry ice once per month to Boston University core lab facility
      - Prior to shipping, slides should be stored in a –80˚C freezer but can be kept in a –20˚C freezer for short-term storage.
8. Document the data point information appropriately on the Case Report Forms:
   - Date and time of surgical lung specimen collection;
   - Number and volume of aliquots prepared;
   - Date and time into –80ºC;
   - Any freeze-thaw that occurs with a sample for any reason;
   - Additional information as specified in the Case Report Forms.

XI. SPUTUM COLLECTION & PROCESSING

Sputum Collection (Site RA to prepare collection material)

Sputum will be processed at Boston University, and is not the responsibility of the sites. However, the DECAMP study coordinator or RA must provide two (2) sputum collection containers with Saccomono's fixative to each participant and instruct the participant on the sputum collection procedure described inAppendix I. To prepare the specimen collection containers, remove each from the manufacturer's packaging and affix a barcoded label on each container (bar code sequence numbers 64 & 65). Remove the screw cap and add 20 cc of Saccomanno's fixative to each container. Replace the cap and twist tightly to securely seal the cap. Return the collection container to the zip closure plastic biohazard bag. Give each participant the plastic zip top biohazard bag with the labeled sputum collection cups, materials to mail the specimens to the Boston University laboratory (postage paid corrugated outer box with UN3373 label) and the instructions for collecting and mailing the sputum specimens.

Sputum Processing (Boston University)

1. **Materials and Equipment**
   - Saccomanno's Fixative*;
   - Benchtop centrifuge;
   - –80ºC freezer.

2. **Procedures**
   1. Pour sputum sample from collection beaker to 50mL conical tube. Rinse residual sputum from collection beaker into 50mL conical with Saccomanno's Fixative bringing the total volume of conical tube to ~45mL*. Discard collection beaker into biohazard waste.
   2. Vortex sample thoroughly promoting separation of mucous and sputum. Saccomanno's Fixative should appear homogenous.
   3. Centrifuge for 10 minutes at 1000rpm at room temperature. Be careful not to disturb tubes after centrifuge.
   4. Carefully discard supernatant. This can be achieved by pouring or sterile pipette. Be careful to remove excess Saccomanno's Fixative and any mucous but <u>not the cell pellet</u>. Some sample's cellular tissue will be hard to see, but cell pellet should rest at bottom of tube.
   5. Add Saccomanno's Fixative to 50mL conical to total volume of ~30mL*.
   6. Vortex sample, centrifuge at 1000rpm and discard supernatant.
   7. Add Saccomanno's Fixative to 50mL conical to total volume of ~20mL*.
   8. Vortex sample, centrifuge at 1000rpm and discard supernatant.
   9. Add Saccomanno's Fixative to 50mL conical to total volume of ~15mL*.
   10. Vortex sample and centrifuge.
   11. Final discard of supernatant should leave ~5ml total volume of Saccomanno's Fixative and cell pellet.
   12. Transfer to 5mL cryopreservation tube and store at –80˚C.
   13. Document the data point information appropriately on the Case Report Forms
       - Date and time of sputum collection
       - Number and volume of aliquots prepared
       - Date and time into –80ºC
       - Any freeze-thaw that occurs with a sample for any reason
       - Additional information as specified in the Case Report Forms

Notes: * Total Saccomanno's fixative required is arbitrary and depends on volume and condition of original sample. Lower volume samples will require less Saccomanno's Fixative, but should still be centrifuged five times. Higher volume samples and/or higher mucous content samples may need to be divided into twoseparate 50 mL tubes and pooled before storage. Lowering the volume is dependent on adequate separation of mucous and cell pellet. Repetition of steps may be necessary to properly wash sputum.

XII. SHIPPING BIOSPECIMENS TO CORE LABORATORY

All biospecimens should not undergo freeze-thaw cycles prior to shipping, so please aliquot volume appropriately. Freezers should have a backup generator or other emergency system. All biospecimens samples must be shipped overnight on dry ice. The approved container must be labeled, appropriately indicating contents using the transmittal worksheets provided in the kits from ACRIN – DECAMP, and be shipped to the designated core laboratory.

Shipping methods should take seasonal temperatures into account, and include the use of extra insulated packaging, cooling agent (cold packs), or dry ice as needed. The standard shipping package for a specimen should include the biohazard bag, placed in a storage box, which is then placed inside a foam-insulated shipping box (bio-shipper).

A. Pathology Tissue Shipments (Ambient)

Some pathology specimens are preserved in paraffin blocks. Prior to shipment, they should be stored in a cool, dark container and be protected from excessive light and temperature to prevent deterioration of the wax and embedded tissue.

The original Specimen Transmittal Form (provided in the kit contents, and always available for download at the ACRIN website | Protocol 4703 Biospecimen Procedures or Protocol 4704 Biospecimen Procedures) must be shipped with the pathology tissue specimens. All corresponding pathology reports will be uploaded to RAVE, and the original pathology reports will be retained in the participant chart.

On the day of shipment, the site's study coordinator will notify the **Boston Medical Center** via e-mail (jackcunn@bu.edu) of the upcoming shipment. The estimated date of arrival and FedEx tracking number must be included in the email.

1. Shipping Materials and Process

The appropriate shipping materials for paraffin tissue specimens are the following:

- Storage boxes for blocks (Fisher; dimensions 5" x 5" x 2");
- Multi-purpose insulated bio-shippers (Thermosafe Bio-Shippers; dimensions 14" x 10" x 14");
- Biohazard bags;
- Shipping labels;
- M3 carton sealing tape;
- Styrofoam peanuts and bubble wrap;
- Cold Packs (to use for all shipments in climates with > 70°F temperature);
- Shipping labels to indicate: "Fragile—Handle With Care" and "Diagnostic Specimens – Not restricted, Packed in Compliance with IATA Packing Instruction 650";

The packing process for shipments includes the following:

- Pack the storage boxes containing the specimens in the shipping container box (14" x 10" x 14");
- Place packing materials such as Styrofoam peanuts and/or bubble wrap in and around the storage boxes to prevent them from shifting during transit;
- Place the Specimen Transmittal Form(s) log inside a zip-lock bag and place the bag inside the insulated shipping container on top of the filler material;
- Close the lids and seal the shipping container with tape;
- Maintain a copy of the transmittal logs at the site.

2. Labeling Shipping Containers

Label each shipping container with the FedEx shipping label to include the following:

1. The participating site's return address, with the study coordinator's name.
2. The Boston University Core Facility address:

Boston University Medical Campus
DECAMP – ACRIN 4703 and/or ACRIN 4704
Attention: Jack Cunningham, Clinical Biospecimen Coordinator
72 East Concord Street, E624
Boston, MA 02118
Phone: 617-358-7097

3. Notice: "Diagnostic Specimens – Not restricted, Packed in Compliance with IATA Packing Instruction 650".
4. Charge the shipping expenses to the 3rd Party Boston University Account provided to DECAMP research staff.

B. Pathology Tissue Shipments (Frozen)

The original Specimen Transmittal Form (provided in the kit contents, and always available for download at the ACRIN website | Protocol 4703 Biospecimen Procedures or Protocol 4704 Biospecimen Procedures) must be shipped with the frozen pathology tissue specimens. All corresponding pathology reports will be uploaded to RAVE, and the original pathology reports will be retained in the participant chart.

On the day of shipment, the site's study coordinator will notify the **Boston Medical Center** via e-mail (jackcunn@bu.edu) of the upcoming shipment. The estimated date of arrival and FedEx tracking number must be included in the email.

1. Shipping Materials and Process

- Multi-purpose insulated bio-shippers (Thermosafe Bio-Shippers; dimensions 14" x 10" x 14");
- Biohazard bags;
- Shipping labels;
- M3 carton sealing tape;
- Styrofoam peanuts and bubble wrap;
- Dry ice;
- Shipping labels to indicate: "Notice: Keep Frozen" use only for **Dry Ice** shipments, Upright arrows, "Diagnostic Specimens – Not restricted, Packed in Compliance with IATA Packing Instruction 650", and "Class 9 – Dry Ice" label; and "Keep Refrigerated" use only for **Cold Pack** shipments.

The packing process for shipments includes the following:

- Place a layer of dry ice on the bottom of the Styrofoam box;
- Put one-half of the 12" x 12" bags of sample vial/tubes into the Styrofoam box on top of the dry ice;
- Layer more dry ice on top of and around the sample bags;
- Put the remaining sample bags into the Styrofoam box on top of the dry ice;
- Layer more dry ice on top of and around the sample bags;
- The amount of dry ice in the shipment should total at least 5 pounds; place packing material on top of the dry ice to fill the box;
- Insert the paper shipping forms into a 12" x 12" bag, and place them on top of the packing material;
- Place the Specimen Transmittal Form(s) log inside a zip-lock bag, and place the bag inside the insulated shipping container on top of the filler material;
- Close the lids and seal the shipping container with tape.

- Maintain a copy of the transmittal logs at the site.

2. Labeling Shipping Containers

Label each shipping container with the FedEx shipping label to include the following:

1. The participating site's return address, with the study coordinator's name.
2. The Boston Medical Center Core Facility address:

   Boston University Medical Campus
   DECAMP – ACRIN 4703 and/or ACRIN 4704
   Attention: Jack Cunningham, Clinical Biospecimen Coordinator
   72 East Concord Street, E624
   Boston, MA 02118
   Phone: 617-358-7097

3. Notice: "Notice: Keep Frozen", "Class 9 – Dry Ice" stickers or "Keep Refrigerated", Upright arrows, and "Diagnostic Specimens – Not restricted, Packed in Compliance with IATA Packing Instruction 650".
4. Charge the shipping expenses to the 3rd Party Boston University Account provided to DECAMP research staff.

C. Cryovials Shipments

The original Specimen Transmittal Form (provided in the kit contents, and always available for download at the ACRIN website | Protocol 4703 Biospecimen Procedures or Protocol 4704 Biospecimen Procedures) must be shipped with the frozen cryovials/tubes specimens.

On the day of shipment, the site's study coordinator will notify the **Boston Medical Center** via e-mail (jackcunn@bu.edu) of the upcoming shipment. The estimated date of arrival and FedEx tracking number must be included in the email.

Specimens for each participant should be packaged separately. Package all vials together according to their type of specimen, each type together in one biohazard bag. Place all bagged cryovials and tubes into a large biohazard bag for a single participant.

1. Shipping Materials and Process

- Storage boxes for cryovial tubes (Fisherbrand, 5 3/4" x 5 3/4" x 4 7/8", Part # 03-395-01, and dividers, Part # 03-395-11);
- Large size zip-locked bags;
- Multi-purpose insulated bio-shippers (Thermosafe Bio-Shippers; dimensions 14" x 10" x 14");
- Biohazard bags;
- M3 carton sealing tape;
- Dry ice or cold packs;
- Shipping labels to indicate: "Notice: Keep Frozen" use only for **Dry Ice** shipments, Upright arrows, "Diagnostic Specimens – Not restricted, Packed in Compliance with IATA Packing Instruction 650", and "Class 9 – Dry Ice" label; and "Keep Refrigerated" use only for **Cold Pack** shipments.

The packing process for **dry ice** shipments includes the following:

- Pack the Styrofoam container with at least 2 inches of dry ice on the bottom;
- Place the single-participant large biohazard bag in the middle of the bio-shipper (you can fit as many as the box allows as long as a single-participant's specimens collection is not split between boxes);
- Pack the Styrofoam container with additional dry-ice until contents are covered;
- Place the Specimen Transmittal Form(s) log for each single participant inside one biohazard bag, and place all bags in the form slot (use as many forms/bags as necessary to cover the

contents of the box to be shipped);
- Close the lids, place all bagged shipping forms on top of the lid, and seal the shipping container with tape.
- Maintain a copy of the transmittal logs at the site.

2. Labeling Shipping Containers

Label each shipping container with the FedEx shipping label to include the following:

1. The participating site's return address, with the site coordinator's name.
2. The Boston Medical Center Core Facility address:

   Boston University Medical Campus
   DECAMP – ACRIN 4703 and/or ACRIN 4704
   72 East Concord Street, E624
   Boston, MA 02118
   Phone: 617-414-4054

3. Notice: "Notice: Keep Frozen" use only for **Dry Ice** shipments, Upright arrows, "Diagnostic Specimens – Not restricted, Packed in Compliance with IATA Packing Instruction 650", and "Class 9 – Dry Ice" label; and "Keep Refrigerated" use only for **Cold Pack** shipments.".
4. Charge the shipping expenses to the 3rd Party Boston University Account provided to DECAMP research staff.

XIII. RECEIPT AND TRACKING OF BIOSPECIMENS

Upon receipt of specimens at the core laboratory, core lab personnel will:

- Confirm that all specimens included in the shipment match the sample transmittal form(s);
- Verify the date of collection and storage conditions;
- Make note of any freeze-thaws that are indicated on the specimen transmittal form(s) and cross-review to make sure all freeze-thaws reported on the Case Report Forms match source documentation.
- Keep specimens frozen; handle only on dry ice while processing.

Any discrepancies must be resolved immediately by contacting the site that sent the specimens. The core lab will be prepared to explain what was found in detail, including dates of reports and specimens type. The site will work to resolve any discrepancies found within the shipment. All issues and resolutions are to be documented at both the receiving core lab and the sending site to ensure consistency of documentation upon audit.

The systems used at the Boston University core lab are FreezerPro and Lockbox, which together have the capability to process a shipping log with audit trail and to include notes regarding shipments as reports are reviewed and queries are resolved/documented.

A. Specimen Login Process

1. Shipment arrives and is immediately put in a –80ºC freezer or other storage as applicable (assumptions that samples are arriving packed in dry ice).
2. Materials' identifiers are logged using the barcode pre-applied to each sample cryovial or supplies as provided to the sites by Boston University – DECAMP.
3. The materials will be returned to the box and stored in –80ºC freezer (assuming all materials in the box is to be stored in the –80ºC freezer).
4. Freezer temperatures are monitored via temperature recorder as well as a central alarm system that is tied into the hospital's main security alert system (alarm goes off; security calls the persons on the call list).
5. The –80ºC freezer is locked via padlock in a secure building with security in lobby and CAS access to only those allowed on the laboratory's floor.
6. The freezer has CO_2 backup and is on hospital emergency power in case of power outage.

APPENDICES

APPENDIX I. Instructions for Participant for Sputum Collection

You have been given two (2) specimen cups containing a special preservative (Saccomono's solution). These cups should be used to collect sputum (phlegm) specimens for the DECAMP trial. You should follow these instructions for your biospecimen collection and submission.

1. Upon rising in the morning, you should thoroughly rinse your mouth with water.
2. You must cough deeply into the sputum cup. It is often easier to produce sputum after your morning shower.
3. Cough on three successive mornings into the same container.
4. At the end of the third date put the date on it and close it.
5. Make sure the cap is screwed on tightly.
6. Place the container in the mailer and put it in a safe place.
7. Store at room temperature.
8. Follow the same procedure for sample collection (1 through 3 above) by coughing three more successive mornings into the second container.
9. After the third day put a date on it and close the lid.
10. Double check that both containers' caps are screwed on tightly.
11. Bring the containers in at your next clinic visit, or mail the two containers at the same time to the Specimen Bank at Boston University.

Again, neither container of sputum needs to be refrigerated prior to mailing, but should be stored at room temperature in a safe place so that they are not accidentally lost.

You may bring the containers into the office for your next clinic visit, or mail them in the postage-paid container that has been provided for you. These containers go through regular mail and can be mailed from your home or post office. Mail the container directly to the Specimen Bank at Boston University.

APPENDIX II. Transmittal Forms

All Biospecimen Transmittal Forms, including individual forms required for submission of biopsy frozen and fixed tissue, are available on protocol-specific Biospecimen Materials pages of the the ACRIN.org web site. These Forms in their most-recent version are available for download at any time.

For submissions associated with ACRIN 4703 DECAMP-1 study, visit:
www.acrin.org/4703_biospecimen.aspx.

For submissions associated with ACRIN 4704 DECAMP-2 study, visit:
www.acrin.org/4704_biospecimen.aspx.

APPENDIX III. Optional Biospecimen Collection Procedures
Buffy Coat Preparation, PBMC Preparation, Urine Processing for Metabolomics

Buffy Coat Preparation (Optional)

1. Materials and Equipment

- Remaining sample from Blood Plasma Preparation;
- Glycerol freezing solution for white blood cells (0.05M Citric acid, sodium salt; 0.02M Sodium phosphate monobasic, monohydrate; 0.02M Sodium phosphate dibasic, anhydrous; 99% glycerol) – this material is not provided in the kit;
- 2 mL Cryovials x 6;
- Serological pipettes of appropriate volumes (sterile);
- –80ºC freezer.

2. Procedure

1. After carefully removing the plasma layer according to the Blood Plasma Preparation section above, carefully remove the buffy coat layer, which will contain a concentrated leukocyte band plus a small portion of the plasma and concentrated RBCs, into a 15 mL centrifuge tube.
2. Using the glycerol freezing solution, the white cell layer should be added and the solution by rocking the tube back and forth. Combining the buffy coat with an equal volume of the glycerol freezing solution is optimal (e.g., 1.5 mL of glycerol freezing solution to 1.5 mL of buffy coat).
3. Aliquot freezing solution and buffy coat mixture into appropriately sized aliquots, attempting not to disturb any of the cell pellet. Transfer buffy coat mixture into a total of 6 cryovials: 4 cryovials (250 µL/vial) and remaining buffy coat into 2 cryovials (500uL/vial). All the sample cryovials are labeled appropriately. At the end of the procedure, check that all aliquot vial caps are secure.
4. After aliquoting the required vials, pipet and save any remaining buffy coat into an extra cryovial. Use a cryovial that is labeled with a barcode.
5. Store vials upright in a labeled specimen box in a –80ºC freezer. All specimens should remain at –80ºC prior to distribution.
6. Document the data point information appropriately on the Case Report Forms:
   - Date and time of blood collection;
   - Number and volume of aliquots prepared;
   - Date and time into –80ºC;
   - Any freeze-thaw that occurs with a sample for any reason;
   - Additional information as specified in the Case Report Forms.

PBMC Preparation (Optional)

For Isolation of PL-PBMC (platelets-less Peripheral Blood Mononuclear Cells), for the DNA Repair OMA Biomarkers Panel**.**

THE ONLY ITEMS PROVIDED IN THE KIT ARE THE 2 YELLOW TOP CITRATE ACD VACUTAINER TUBES FOR THE BLOOD COLLECTION; ALL OTHER SUPPLIES WILL NEED TO BE PROVIDED BY SITE.

1. **Materials and Equipment**
   - 2x ACD Blood Collection Tubes Yellow conventional closure. ACD Solution A of trisodium citrate, 22.0 g / L; citric acid, 8.0 g / L; and dextrose 24.5 g / L, 1.5mL;
   - UNI-SEP test tubes for density gradient fractionation of human PBMC (from NOVA*med* ; cat#U10);
   - Sterile PBS: Dulbecco's phosphate buffered saline, pH 7.4;
   - PBS + 2 mM EDTA: Dulbecco's phosphate buffered saline, pH 7.4 supplemented with 2 mM EDTA;
   - 10x Red Blood Cell Lysis buffer (10x RBCL buffer), composed of 1.55 M NH_4Cl, 0.1M $KHCO_3$ and 1 mM EDTA. Solution for daily use is stored at 4˚C.$KHCO_3$ and 1 mM EDTA;
   - 1x Red Blood Cell Lysis buffer (1x RBCL buffer), freshly prepared by mixing 5 ml 10x RBCLbuffer, 5 ml PBS and 40 ml sterile deionized water (total volume 50 ml);
   - LTGO buffer: 50 mM Tris.HCl (pH 7.1), 1 mM EDTA, 0.5 mM spermidine, 0.1 mM spermine, and a protease inhibitor cocktail (Sigma, cat# P8340; 0.5 mL in a final volume of 50 mL LTGO buffer). Divide into 10 mL samples, and store at –20˚C;
   - Ficoll-Paque;
   - Benchtop centrifuge with swing-out rotor and appropriate carriers;
   - Optional Blood counter (i.e., Cobas Micros, Roche Diagnostic System);
   - –80ºC freezer;
   - 5 mL cryovials x 5.

2. **Procedure**
   1. ***Blood samples should be processed within 2 hours of blood collection.***
   2. Collect 8.5 mL of blood samples into two ACD vacutainers each; gently mix the blood by inverting the tubes 8 to 10 times. Store the blood samples at room temperature until processing.
   3. Transfer 100 µL of whole blood to a counting test tube and analyze it in a blood counter (this step is not obligatory, but it is recommended since it provides info on the blood samples).
   4. At 20˚C, centrifuge each 8.5 mL vacutainer tube at 400 g. Stop without brakes.
   5. Combine the blood from the 2 tubes into a 50 mL test tube and dilute it to a final volume of 35 mL with PBS+2mM EDTA. Gently mix by inverting the test tube 5 to 10 times.
   6. Before adding the diluted blood to the UNI-SEP test tube, first, spin the UNI-SEP test tubes for 1 minute at 400 g at 20˚C to make sure that the Ficoll is in the lower compartment of the test tube.
   7. Add 35 mL of the diluted blood sample to the UNI-SEP test tube; secure its cap and centrifuge (20˚C) at 1000 g for 30 minutes using a swinging-bucket rotor. Stop without brakes.
   8. Using a 5 mL pipette to collect and transfer the PBMC layer into a 15 mL conical test tube. If the volume of the cells is larger than 5 to 6 mL, divide the cells to 2x 15 mL conical test tubes. (At least 2 volumes of PBS+2mM EDTA must be added to the cells in order to dilute the ficoll and enable efficient sedimentation of the cells).
   9. Washing step: Add PBS+2mM EDTA to a final volume of 15 mL, mix gently by inverting the test tubes 5 times, then centrifuge (10˚C) at 200 g for 10 minutes. After centrifugation, remove the supernatant by suction, and discard it to a biohazard container.
   10. Lysis of Red blood cells: Incubate the PBMC at room temperature in 5 mL 1xRBCL buffer for exactly 4 minutes. To facilitate the suspension of the lymphocytes, this step is done in two steps: first the pellet of both tubes is combined (if it was split) and suspended in 1 mL 1xRBCL buffer. Additional 4 mLs of 1xRBCL buffer are added and the mixture is further suspended by pulling-up

and down the suspension in the pipette. The 4 minutes of the incubation time starts upon addition of the 1 mL 1xRBCL buffer.

11. At the end of the 4 minutes incubation time, add 10 mL PBS+2mM EDTA to the test tube, cap it and mix gently by inverting the test tubes 5 times, then centrifuge (10˚C) at 200 g for 10 minutes. After centrifugation, remove the supernatant by suction and discard it to a biohazard container.
12. Wash with 14 mL PBS+2mM EDTA. Again the suspension is done in two steps: first the pellet is suspended in 1 mL PBS+2mM EDTA, and then an additional 13 mL PBS+2mM EDTA are added. Mix gently by inverting the test tubes 5 times and centrifuge (10˚C) at 200 g for 10 minutes. After centrifugation, remove the supernatant by suction and discard it to a biohazard container.
13. Wash with 14 mL PBS+2mM EDTA. Again the suspension is done in two steps: first the pellet is suspended in 1 mL PBS+2mM EDTA, and then an additional 13 mL PBS+2mM EDTA are added. Mix gently by inverting the test tubes 5 times and centrifuge (10˚C) at 200 g for 10 minutes. After centrifugation, remove the supernatant by suction and discard it to a biohazard container.
14. Cell counting: Add 1 mL PBS (without EDTA) and suspend the PBMC to homogeneity. Then dilute 50 µl of the cell suspension with 10 mL PBS and suspend the PBMC to homogeneity. Analyze it in a cell counter.
15. Place 2.5 x 10^6 PBMCs in 1 or more eppendorf test tubes (use the coulter results as a guide), and centrifuge at 5000 rpm for 4 minutes at room temperature. Adjust PBMC concentration to 50,000 cells/µL in LTGO buffer.
16. Transfer the adjusted PBMC into the pre-labeled 5 mL vials x5.
17. Incubate the cells 30 minutes on ice.
18. Flash freeze the cells in liquid nitrogen and then transfer to a –80°C freezer. Alternate flash freezing procedure: 100% isopropyl (or methanol) with dry ice bath to snap freeze the tissue if liquid nitrogen is not available.
19. Document the data point information appropriately on the Case Report Forms:
    - Date and time of blood collection;
    - Date and time of beginning of blood processing;
    - PL-PBMC count;
    - Number of aliquots and number of cells and LTGO volume of each aliquot;
    - Number and volume of aliquots prepared;
    - Date and time into –80ºC;
    - Any freeze-thaw that occurs with a sample for any reason;
    - Any variations or deviations from these procedures, problems, or issues.

Urine Processing for Metabolomics Study (Optional)

1. Materials and Equipment

THE ONLY ITEM PROVIDED IN THE KIT IS A NON-COATED URINE CUP FOR THE OPTIONAL URINE COLLECTION; ALL OTHER SUPPLIES WILL NEED TO BE PROVIDED BY SITE.

- Chenomx standard solution;
- 5 mL cryovials x 6;
- Sterile conical tubes (15 mL);
- Approved urine specimen cups (100 mL) coated with sodium azide (NaN_3);
- –80ºC freezer.

2. Procedure

1. In preparation for sample collection, coat urine specimen cups (100 mL) with sodium azide (NaN_3) in advance, by adding 100 μL of 10% NaN_3 and allowing it to dry at room temperature. Prepared cups can be stored dry in a zip-lock plastic bag at 4°C for up to 6 months.
2. Instruct participants to first clean the urethral area with a castile soap towelette.
3. Instruct participants to collect 50 mL of urine midstream into a clean approved container. Excess urine should be voided into the toilet. This method of collection should be conducted in the clinic.
4. Seal immediately and place on ice or in the refrigerator (4ºC). Record date and time of collection.
5. Reserve 10 mL of the total collected urine volume in a sterile conical tube and refrigerate immediately.
6. Check and record urine pH using a pH meter and, if needed, carefully adjust the urine pH to ~7.0 ± 0.2 using NaOH (0.1 M) or HCl (0.1 M). Please try to take care not to overshoot or repeatedly adjust pH, as high salt concentrations (400 mM or more) can interfere with spectra collection.
7. Keep tubes and samples on ice while generating sample aliquots by allocating 1 mL of urine into a labeled sterile Eppendorf tube; generate up to 5 aliquots/patient.
8. Centrifuge each tube at 14,000 rpm (~10,000 *g*) in a refrigerated microfuge (4ºC) for 5 minutes.
9. Remove 900 μL of the supernatant and transfer to a sterile pre-labeled 2 mL cryovial. Take care not to disturb the bottom of the tube whether there is a visible pellet or not. Discard the pellet and ~100μL of sample above it.
10. Add 100 μL of Chenomx standard solution to each aliquot.
11. Freeze aliquots at –80°C.
12. Document the data point information appropriately on the Case Report Forms:
    - Date and time of urine collection;
    - Number and volume of aliquots prepared;
    - Date and time into –80ºC;
    - Any freeze-thaw that occurs with a sample for any reason;
    - Additional information as specified in the Case Report Form.

Nasal Epithelium Collection for Single Cells (Optional)

An optional third nasal brush for single cell sorting may be taken at select DECAMP sites: Walter Reed, Boston University Medical Center and University of California Los Angeles.

1. Materials and Equipment

- Sort Buffer: 1% FBS in PBS;
- Wash Buffer: PBS;
- 10X RBC Lysis ;
- dH2O;
- Cryostor Media;
- 0.25% Trypsin:EDTA;
- 100mm cell culture plate x 1;
- 6 well cell culture plate x 1;
- 50mL conical tube x 2;
- 2mL cryovial x 2;
- Mr. Frosty isopropyl alcohol chamber (FisherSci);
- Benchtop centrifuge;
- –80ºC freezer.

2. Procedure

1. **Brush Preparation** (in 1mL Sort Buffer)
   a) Rinse each brush (after clipping the tip from the entire brush) with 5-10mL Sort Buffer into a 100mm plate. Please note, you should use the pipettor to wash up and down the brush tip to get maximal cell recovery.
   b) Rinse out the tube holding the brush twice with 1 mL Sort Buffer.
   c) Transfer contents of plate to a 50mL tube.
   d) Rinse off plate three times with 5 mL Sort Buffer, then transfer to tube.
   e) Centrifuge at 2000 rpm for 5 minutes.
   f) Wash with 20mL PBS.
   g) Centrifuge at 2000 rpm for 5 minutes.

2. Trypsinization

   a) Add 0.75mL of 0.25% Trypsin:EDTA, then transfer to 6 well plate.
   b) Add another 0.75 mL of 0.25% Trypsin:EDTA, then transfer remainder to plate.
   c) Incubate for 5 minutes, then mix cells with 1mL pipet followed by 200uL followed by 20uL pipet to mechanically dissociate cells. (Repeat up to 3 times, for a maximum total incubation time of 20 minutes.) ****Please note:** stop the trypsinization step when you see most of the cells are in single cell suspension. Over-trypsinization may damage the cells for down-stream processing and affect sequencing quality.
   d) Add 4 mL Sort Buffer and mix well to inactivate Trypsin.
   e) Pipet cells through 40um strainer into 50mL tube.
   f) Wash well with 1mL Sort Buffer and pipet through strainer until about 10mL total.
   g) Centrifuge for 5 minutes at 2000 rpm.

3. RBC Lysis

   a) Prepare 1X RBC Lysis Buffer for 1 sample (5mL).
      a. 0.5mL 10X RBC Lysis + 4.5mL UP dH2O
   b) Resuspend cells in 5mL 1X RBC Lysis Buffer
   c) Put tube on ice for 2 minutes, shaking gently at 1 minute.
      a. ****Please note:** If you started with a very bloody sample, you may want to incubate for an extra minute.
   d) Add 20mL PBS to stop reaction.

e) Centrifuge for 5 minutes at 2000 rpm (re-centrifuge if needed).
f) Resuspend cells in 50uL Sort Buffer.

4. Cryopreservation: re-suspend pellets in Cryostor media and freeze slowly in a Mr. Frosty isopropyl alcohol chamber (FisherSci) at −80 °C. This procedure allows us to achieve a rate of cooling of −1 °C/minute.