The SPIROMICS Bronchoscopy Substudy Study Design and Manual of Procedures

Version 5.04
September 15, 2015

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STUDY DESIGN

1.0.1 Sample Size

A subgroup of 50 subjects per site (total n=300)

1.0.2 Bronchoscopy Enrollment Strata

Our strata enrollment will deviate slightly from that of the parent study because we have two additional specific goals: 1) to exclude subjects with very low FEV1 for safety (<30% predicted) and 2) to oversample non-smoking healthy controls and smokers with normal lung function to ensure a large enough control groups for sample analysis.

	Non-Smokers	**Smokers**	**Mild/Moderate COPD**	**Severe COPD**
Smoking Status	< 1 pack year	> 20 pack years	> 20 pack years	> 20 pack years
Lung Function	Pre-bronchodilator $FEV_1/FVC > .7$ and FVC>LLN	Post bronchodilator $FEV_1/FVC >$ .7 and FVC>LLN,	Post-bronchodilator $FEV_1/FVC < .7$ and $FEV_1 > 50\%$ pred.	Post-bronchodilator $FEV_1/FVC < .7$ and $FEV_1 < 50\%$ pred but > 30% pred.
Sample Size	N = 60 (20%)	N = 100 (33%)	N = 120 (40%)	N = 20 (7%)

1.0.3 Visits

We propose 2 separate visits. The first will be for sputum induction in order to obtain a sputum sample for immunophenotyping. The second visit will be for bronchoscopy and will take place 2-12 weeks after the sputum visit. The bronchoscopy substudy visits can be completed at any time and do not need to be tied to a main study visit. Participants who have completed all main study visits can also be enrolled in the Bronchoscopy Substudy.

1.0.4 Samples

- Blood for immunophenotyping and CBC with differential cell count
- Tongue Scraping
- Oral Rinse
- Saline (microbiome control)
- Saline suctioned through scope (microbiome control)
- Bronchial Wash
- BAL
- Epithelial Brushes (large airway brushings required, distal airway brushings optional)
- Endobronchial Biopsies

1.0.5 Start-up Procedures

NOTE: The first five bronchoscopies completed at each site should be in subjects with a post-bronchodilator FEV1 >50% predicted. The data from these first five bronchoscopies must be reviewed by the Bronchoscopy Subcommittee before the site is approved to proceed with subjects with lower FEV1.

1.1. INCLUSION/EXCLUSION

Inclusions:

- Subjects enrolled in the SPIROMICS who consent to a research bronchoscopy and who meet all local requirements for a bronchoscopy (e.g., any laboratory tests that are required by institutional policy to be administered prior to a bronchoscopy), are eligible for this substudy.

Exclusions:

- Age>80
- History of cardiac disease or other comorbid condition severe enough to significantly increase risks based on investigator discretion.
- Pao2/Sao2 that qualifies them for supplementary oxygen at rest (PaO2< 60 or SaO2<88%)
- post-BD FEV1<30% predicted
- Use of anticoagulation (patients on warfarin or clopidogrel will be excluded, patients on aspirin alone can be studied (3) even with concurrent use)

1.2. SPECIAL CONSIDERATIONS IN COPD

The presence of COPD has been shown to increase the complication rate of bronchoscopy (where FEV1/FVC <50% or FEV1 <1 litre and FEV1/FVC <69%) (1). A complication rate of 2-5% occurred in the patients with severe COPD (1, 2) compared with 0.6% in those with normal lung function. The use of sedation in patients with severe COPD has increased risk relating to potential carbon dioxide retention.

2 SUPPLIES AND EQUIPMENT

2.1 Oral rinse and tongue scraping

1. 1 10 cc bottle of sterile saline
2. 1 sterile collection cup (90 mL)
   a. ORAL (will include both tongue scraping and oral rinse) + 20 cc of RNA Later
3. 3 clean disposable tongue scrapers

2.2 Saline alone and Suctioned through the Scope (microbiome controls)

1. Sterile 20cc syringe
2. Sterile specimen cup
3. 2 x 15 mL sterile conical tubes, each containing 10mL of RNALater

2.3 Bronchoalveolar Lavage and Wash

1. Sterile polypropylene conical centrifuge tubes (50 mL and 15 mL)
2. Frosted Plus slides and coverslips
3. Shandon Diff Quick stain kit (# 9990700)
4. Turks solution (1% glacial acetic acid and 0.01% gentian violet in distilled H_2O)
5. Hemocytometer
6. Cell counter
7. Cytoseal 60 (VWR cat # 48212-154)
8. 500 mL of sterile normal saline (0.9%)
9. Microscope
10. Centrifuge (with appropriate rotors) for 50 mL and 15 mL tubes of BAL
11. Shandon cytocentrifuge, metal slide holders, cytofunnels and filter cards [Shandon or similar (800) 245-6212].
12. Common laboratory supplies: Pipette aid, graduated pipettes, Pipetmen and pipette tips, etc.
13. Sterile beaker for mixing/pooling lavage fluid
14. Sterile slip-tip syringes for saline aliquots (30cc syringes for wash, 50cc syringes for lavage)
15. RNase-free PBS
16. RNALater™
17. 700µl QIAzol lysis solution x 2

2.4 Epithelial Brushings

1. For microbiome analyses, protected brushes will be BARD Disposable Microbiology Brushes (ConMed, Catalog # 130, phone # (800) 448-6506).
2. For each of these microbiology brushes, prepare a 2.0 mL aliquots filled with 1.5 mL of RNALater™ sitting on ice.
3. Wire CuttersFor large airway epithelial brushings, we will use disposable cytology brushes (ConMed, Catalog # 149, 3mm diameter x 11mm long, phone # (800) 448-6506).
4. For small airway epithelial brushings we will use Kimberly-Clark cytology brushes, 2.0 mm diameter, reference #60311 (different from the large airway cytology brush)
5. Small 2.0 mL screw top tubes (sterile RNAase free)
6. RNase-Free PBS
7. Wire Cutters
8. Vortex
9. 15 mL Conical Tubes (Falcon or similar)
10. 700µl QIAzol lysis solution

2.5 Endobronchial Biopsy

1. Disposable forceps (considering Olympus Disposable Biopsy Forceps #FB-231D.A, required working channel 2.0mm, length 115cm, cup opening 5.0mm)
2. 700µl QIAzol lysis solution
3. RNase-Free PBS
4. Petri dish
5. 25 gauge needle and 1cc syringe
6. 10% formalin
7. 70% ethanol
8. Liquid nitrogen and thermos

2.6 Immunophenotyping BAL

1. Immunophenotyping BAL assay tubes with pre-aliquoted antibodies (shipped from immunophenotyping core and stored at 4°C wrapped entirely in tinfoil to prevent photobleaching.)
2. Common laboratory supplies: pipettes and pipette tips, tinfoil
3. Swinging bucket centrifuge for 5 ml tubes with biocontainment lids and capacity to refrigerate
4. Staining Buffer, w/ FBS (BD Biosciences, #554656)
5. Formaldehyde, 20% (Tousimis, #1008A); 20% formaldehyde is shipped in glass vials. After opening, store remaining 20% formaldehyde in 15 ml conical tube at 4°C and wrap in tinfoil to protect from light
6. Laboratory rotator or shaker
7. Immunophenotyping Shipper - used to receive and send assays from and to immunophenotyping core (consists of cardboard shipper box, polystyrene cooler, cold pack, eggshell foam)

2.7 Immunophenotyping blood

1. Immunophenotyping blood assay tubes with pre-aliquoted antibodies (shipped from immunophenotyping core and stored at 4°C wrapped entirely in tinfoil to prevent photobleaching).
2. Common laboratory supplies: pipettes and pipette tips, tinfoil, distilled water
3. Swinging bucket centrifuge for 5 ml tubes with biocontainment lids and capacity to refrigerate
4. Staining Buffer, w/ FBS (BD Biosciences, #554656)
5. Blood collection tube containing heparin
6. Formaldehyde, 20% (Tousimis, #1008A); 20% formaldehyde is shipped in glass vials. After opening, store remaining 20% formaldehyde in 15 ml conical tube at 4°C and wrap in tinfoil to protect from light
7. BD Pharm Lyse, 10x (BD Biosciences, #555899)
8. Laboratory rotator or shaker

9. Immunophenotyping Shipper - used to receive and send assays from and to Immunophenotyping Core (consists of cardboard shipper box, polystyrene cooler, cold pack, eggshell foam)

2.8 Immunophenotyping Sputum

1. Immunophenotyping sputum assay tubes with pre-aliquoted antibodies (shipped from immunophenotyping core and stored at 4°C wrapped entirely in tinfoil to prevent photobleaching).
2. Cryovial for microbiome aliquot
3. Common laboratory supplies: pipettes and pipette tips, tinfoil, distilled water, PBS
4. Swinging bucket centrifuge for 5 ml tubes with biocontainment lids and capacity to refrigerate
5. Staining Buffer, w/ FBS (BD Biosciences, #554656)
6. Formaldehyde, 20% (Tousimis, #1008A); 20% formaldehyde is shipped in glass vials. After opening, store remaining 20% formaldehyde in 15 ml conical tube at 4°C and wrap in tinfoil to protect from light
7. Sputolysin (Calbiochem, #560000)
8. Mesh filter paper
9. Laboratory rotator or shaker
10. Water bath
11. Vortex
12. Digital scale or balance
13. Immunophenotyping Shipper - used to receive and send assays from and to immunophenotyping core (consists of cardboard shipper box, polystyrene cooler, cold pack, eggshell foam)

2.9 SOLUTIONS LISTING

2.9.1 RNase Free PBS for brushing sampling

Materials:

1. 1 pre-packaged powdered 10 X PBS Mix: (Sigma-Aldrich: # P3813-10PAK)
2. 1 liter Nuclease/RNase Free Water - non-DEPC treated: (Ambion: # AM9932)

Directions:

Sites are provided with liquid PBS in the appropriate concentration. If making liquid PBS from powder:

1. Add 1 package 10X PBS powder to 1 liter Nuclease/RNase-free Water
2. Mix well
3. Aliquot if necessary to avoid contamination from frequent use
4. Treat as sterile solution

5. Avoid contamination by pipetting carefully and wearing gloves

Using liquid PBS:

1. Treat as sterile solution
2. Avoid contamination by pipetting carefully and wearing glove
3. Aliquot sample prep tubes with 1 mL PBS prior to each bronchoscopy and store on ice

2.9.2 RNALater for microbiome analyses
RNALater should be pre-aliquoted in a biohazard hood PRIOR to collection. Use pipet aid and sterile 10 mL pipet tips. Record date the RNALater was opened on bottle. It can be used for up to 1 year and longer if no white precipitate forms. If a precipitate does form, heat it to 37 degrees C and agitate to re-dissolve it. Please store RNALater at room temperature (25 degrees C) or at 4 degrees C. RNALater is used to preserve the integrity of RNA and DNA. For best results, please mix sample with RNALater immediately. Samples with RNALater can be stored at room temperature (25 degrees C) for 24 hours, at 4 degrees C for 4 weeks, and at -80 degrees C indefinitely. RNALater can be safely discarded down the sink and flushed with water. Note: it is known to react with hypochlorite solution (bleach).

a. ORAL sample: 90 mL orange topped collection cup containing 20 mL of RNALater
b. Saline alone and saline suctioned through scope: 2x15mL sterile conical tubes each containing 10mL of RNAlater
c. Bronchial wash: 50 mL sterile conical tube containing 9 mL RNALater
d. BAL: 50 mL sterile conical tube containing 9 mL RNALater
e. Microbiological brushes: three 2.0 mL microtubes containing 1.5 mL of RNALater™ sitting on ice

3 PROCEDURES

3.0 RECRUITMENT AND CONSENT

3.0.1 Recruitment

Each site will recruit approximately 10 healthy controls (stratum 1), 10 smokers without COPD (stratum 2), 23-24 mild-moderate COPD (stratum 3), and 6-7 severe COPD (stratum 4) participants.

Participants enrolled in main SPIROMICS can be recruited at any time for the Bronchoscopy Substudy, including between main study visits and after completion of all main study visits. As part of the main study consent, participants are asked whether they would like to be contacted regarding participation in other studies. Participants who indicate they did not wish to be contacted should not be approached about participating in

the bronchoscopy substudy. Further, coordinators are encouraged to wait until after a participant eligibility and study stratum has been determined before inviting subjects to participate in this substudy.

3.0.2 Consent

Study coordinators should describe the bronchoscopy substudy to the participant. If the subject is interested in participating in the substudy, the coordinator should provide the participant with a copy of the consent form. The coordinator should carefully review, although not read, the consent form with the participant to make sure he or she fully understands the substudy procedures and risks.

3.1 INITIAL VISIT AND SCREENING

Once the participant has consented the coordinator should administer the Bronchoscopy Inclusion/Exclusion Criteria Form (BIE), to confirm the participant meets the inclusion/exclusion criteria. Once eligibility is confirmed the participant should meet with a study physician, preferably the bronchoscopist, for a brief history and physical to confirm the subject is healthy enough to participate in the substudy. The physician should review the substudy procedures and risks with the participant and address any concerns he or she may have.

If the physician agrees the participant is eligible to continue in the substudy the coordinator should proceed with scheduling the visits. If any special blood work is required by site regulation it should be drawn at this point.

3.2 SCHEDULING THE SUBSTUDY VISITS

The bronchoscopy substudy involves two separate visits: one for sputum induction and one for the bronchoscopy. The first visit is for a sputum induction. The second visit, bronchoscopy, should be scheduled at least 2 weeks but not more than 12 weeks after the sputum induction visit. Because of the tight timeline coordinators are encouraged to schedule both visits at the same time.

In addition to the timing of the visits in relation to each other, study coordinators need to allow enough time for the Immunophenotyping Core to prepare and ship the pre-made antibody aliquots. For this reason, coordinators should request the pre-made antibodies from the Immunophenotyping Core at least one week prior to each visit (i.e., the sputum induction visit and the bronchoscopy substudy visit). Please see details on this scheduling in Section 3.3.

3.3 PROCEDURES FOR THE PRE-MADE ANTIBODY ALIQUOTS FOR IMMUNOPHENOTYPING SAMPLES

1. **One or more weeks prior to sputum induction:** Once you have scheduled the sputum induction and bronchoscopy substudy visits, simultaneously send an email to

Christine Freeman (cmfreema@umich.edu), Valerie Stolberg (vstolberg@yahoo.com), Sean Crudgington (scrudgin@umich.edu) AND Jeff Curtis (jlcurtis@umich.edu) with the date of the sputum induction and bronchoscopy visits. If you do not receive an email response within 24 hours call 734-845-5078 or 734-546-6059 to confirm receipt of your email.

2. **Three days prior to sputum induction**: (1) If you have not received an email stating that the pre-made antibody aliquots for the sputum induction have been shipped along with the tracking information for said aliquots, please call 734-845-5078 or 734-546-6059. (2) If it is one or two days prior to the sputum induction visit and you can see from the tracking number that the antibodies are stuck somewhere in shipping, please first contact FedEx by calling 1-800-GoFedEx (1-800-463-3339).

   If you do not receive the pre-made antibody aliquots you will need to reschedule the sputum induction visit (the same does not apply to the bronchoscopy visit).

3. **After sputum induction:** If samples were obtained follow the shipping procedures describe in Section 6.2. If samples were not obtained, please email Christine Freeman (cmfreema@umich.edu), Valerie Stolberg (vstolberg@yahoo.com), Sean Crudgington (scrudgin@umich.edu) AND Jeff Curtis (jlcurtis@umich.edu) to let them know there will be no specimens for analysis.

4. **One week prior to bronchoscopy visit:** Send a reminder email to Christine Freeman (cmfreema@umich.edu), Valerie Stolberg (vstolberg@yahoo.com) , Sean Crudgington (scrudgin@umich.edu) AND Jeff Curtis (jlcurtis@umich.edu) with the date of the bronchoscopy visit. If you do not receive a response within 24 hours call 734-845-5078 or 734-546-6059.

5. **Three days prior to bronchoscopy visit**: (1) If you have not received an email stating that the pre-made antibody aliquots for the blood and BAL have been shipped along with the tracking information for said aliquots, please call 734-845-5078 or 734-546-6059. (2) If it is one or two days prior to the bronchoscopy visit and you can see from the tracking number that the antibodies are stuck somewhere in shipping, first contact FedEx by calling 1-800-GoFedEx (1-800-463-3339).

   If you do not receive the pre-made antibody aliquots **do not** reschedule the bronchoscopy visit.

6. **After bronchoscopy visit:** If samples were obtained follow the shipping procedures describe in Section 6.2. If either blood or BAL immunophenotyping samples were not obtained, please email Christine Freeman (cmfreema@umich.edu), Valerie Stolberg (vstolberg@yahoo.com) , Sean Crudgington (scrudgin@umich.edu) AND Jeff Curtis (jlcurtis@umich.edu) to let them know there will be no specimens for analysis.

3.4 SPUTUM INDUCTION

Sputum should be induced as described in MOP 5 – Sputum Induction and Processing. This visit will occur at a separate visit that will precede bronchoscopy by at least two weeks but no more than four weeks.

3.4.1 Sputum Sample Processing (Immunophenotyping)

1. Sputum should be induced as described in MOP 5 – Sputum Induction and Processing
2. Pour entire sputum sample into Petri dish
3. Weigh an empty 50 ml tube and zero the balance
4. Transfer the non-liquid portion of the sputum to the 50 ml tube and measure weight in grams
5. If the sample weighs < 1.3 grams, proceed directly to step 6. If sample weighs ≥ 1.3 grams, transfer 0.3 grams into separate cryovial for microbiome analysis and store at -80°C, prior to shipping (refer to Section 6.1). Please refer to section 3.4.1.1 for details on sputum microbiome specimen storage. Proceed with step 6 to process the remaining sample.
6. Make up 1x Sputolysin: combine 1 ml of Sputolysin with 9 mLs of distilled water and swirl gently to dissolve crystals
7. Multiply the weight of the sputum by 2 and add that many mLs of 1x Sputolysin to the 50 ml tube (e.g. if sputum weighs 1 gram, add 2 mLs 1x Sputolysin)
8. Incubate in 37°C water bath for approximately 20 minutes (or until the sputum appears viscous) with vortexing every 5 minutes
9. Dilute with an equal volume of PBS (e.g. if 2mLs 1x Sputolysin was used, dilute with 2mLs PBS)
10. Filter through nylon mesh filter into fresh 50 ml tube
11. Centrifuge at 300 x g for 5 minutes
12. Decant supernatant
13. Resuspend cells in 1.0 ml cold Stain Buffer
14. Briefly centrifuge immunophenotyping sputum assay antibody tubes (supplied by immunophenotyping core) at 300 x g for 2 minutes.
15. Uncap tubes and add 100 µl of sputum sample to each tube. Gently mix tube by swirling.
16. Wrap rack with tinfoil to protect from light
17. Incubate on shaker for 25 minutes at room temperature
18. Add 2 mLs of cold Stain Buffer to each tube. Gently mix tube by swirling.
19. Centrifuge at 300 x g for 5 minutes
20. Decant supernatant
21. Prepare 2% formaldehyde solution: Combine 0.2 mLs 20% formaldehyde with 1.8 mLs cold Stain Buffer. (Note: 2% formaldehyde should not be made more than 30 minutes in advance and should be discarded immediately after use. It cannot be saved for future use)

22. Add 200 µl of 2% formaldehyde to each tube. Pipette up and down to mix.
23. Replace caps on tubes; they should snap firmly into place (should hear two clicks)
24. Wrap rack in a new sheet of tinfoil – NOTE: Your samples should be in 15ml tubes and NOT 50ml tubes at this point.
25. Store at 4°C until ready to be shipped
26. Ship sample to Immunophenotyping Core using the procedures described in Section 6.2

3.4.1.1 Sputum Microbiome Sample Storage

Set aside a freezer box for microbiome sputum sample storage. Samples from multiple participants will be stored in a single box. In addition the labeling the specimen aliquot, place sample labels on both the bottom and the lid of the freezer box for each specimen added to the container. Sputum microbiome samples should be shipped to the BSP when sending other Bronchoscopy Substudy samples (see Section 6.1 for shipping details). Coordinators do not need to wait for a complete box to ship these samples.

3.5 BRONCHOSCOPY

The procedures outlined herein are meant to serve as *guidelines*, with room for institutional flexibility for issues including sedation and topical anesthesia methods, route of bronchoscope introduction and subject monitoring.

3.5.1 Pre-Bronchoscopy Visit Patient Instructions

The patients should be advised to dress comfortably. They should have nothing to eat or drink (including water) for at least 8 hours prior to the procedure. An adult must be available to drive and accompany subject home after the procedure. Other exclusion criteria are noted above.

3.5.2 Pre-Bronchoscopy Set Up

Set up oxygen. Attach humidifier bottle to oxygen and nasal cannulae to the humidifier bottle. Set up primary and secondary suction. Primary suction set to full vacuum to provide suction for the bronchoscope. Secondary suction set to full vacuum to aspirate the oro-pharynx. Turn both on and verify adequate suction.

Set up the bronchoscope. Connect two 33 inch extension sets together and attach the male end into the suction port of the bronchoscope and the female end to the suction trap. Attach the "T" adapter to the instillation port of the bronchoscope. Cap the top port of the "T" adapter with the rubber cap and insert the male end of a single 33 inch extension set into the side port of the "T" adaptor. Attach a stopcock to the female end of this single extension set (instilling line).

Set up the pulse oximeter, automatic sphygmomanometer, and light source for the bronchoscope. Check all equipment for its operational status.

Prepare the upper airway anesthesia tray using 1%, 2%, and/or 4% lidocaine (per center-specific protocols).

IV set up. Assemble a 250 – 500 mL 0.9% saline bag, IV extension set with at least one injection port, 22G angiocath, 1/4" tape, tegaderm, alcohol swipe, 2" X 2" gauze, sharps container, tourniquet and gloves.

3.5.3 Prepare for bronchoalveolar and endobronchial biopsy harvesting

Bronchoalveolar lavage: Set up the water bath (fill with distilled water and warm to 37 degrees C), warm a 500 mL 0.9% saline bag to 37degrees C, and place specimen trap(s) on ice. A 60 cc syringe is used to draw up to instill the warmed saline fluid. Lavage return is collected on ice.

Endobronchial Biopsy: Forceps description: (Olympus #FB-231D.A, required working channel 2.0mm, length 115cm, cup opening 5.0mm).

Prepare a 1:20,000 epinephrine solution. Add 1 mL 1:1000 epinephrine to 19 mL 0.9% saline. Assemble fixatives and collection containers, 26 gauge needle with syringe.

3.5.4 Pre-Bronchoscopy Assessment/Procedures

Measure and record vital signs (blood pressure, heart rate, oxygen saturation, respiratory rate, and temperature), a rhythm ECG, and verify proper NPO status.

FEV_1 should be measured before and 15 minutes after delivering 4 puffs of albuterol. Sites may use a Piko meter or spirometer to take this measurement. Sites are not required to use the study provided spirometer. <u>If the participant's post-bronchodilator FEV_1 on the day of the bronchoscopy is ≤ 30% predicted the bronchoscopy will need to be canceled or rescheduled.</u>

Conduct a pre-bronchoscopy history and physical, collect a modified aldrete score, review the post-bronchoscopy instructions, and the plan for conscious sedation. Place an IV line.

3.5.5 Immunophenotyping Blood Protocol

Three tubes of blood (a 10ml green top, a 9.5ml lavender top (EDTA) tube and a CBC tube) should be drawn during the bronchoscopy visit (at the time of the IV placement). The green top tube will be used for immunophenotyping. The 10ml lavender top tube will be used for plasma aliquots. The CBC with differential will allow for normalization of immunophenotyping data. The CBC will be conducted at the local, clinical lab. The preferred protocol will be to draw the blood from the IV itself at the time of the

placement. If that is not possible, a separate phlebotomy will be acceptable but this information should be recorded.

Plasma aliquots from the lavender top tube

1. The 9.5 ml BD Vacutainer® plastic EDTA tube (lavendar top) should be processed for plasma within two hours
2. Use the main SPIROMICS study protocol for preparing, aliquoting, storing and shipping these plasma aliquots. Plasma aliquots should be stored after bronchoscopy specimen samples in the specimen freezer box.

Immunophenotyping with the green top tube

1. Use the 10 ml green top heparin plasma tube with blood collected for immunophenotyping (described in MOP 4). Invert gently 8 times.
2. Briefly centrifuge immunophenotyping blood assay antibody tubes (supplied by immunophenotyping core) at 300 x g for 2 minutes and uncap tubes
3. Add 100 µl of undiluted blood to each tube. Gently mix tube by swirling.
4. Wrap rack with tinfoil to protect from light
5. Incubate on shaker for 25 minutes at room temperature
6. Make up 1x lysis buffer: Combine 36 mLs distilled water with 4 mLs 10x BD Pharm Lyse
7. Let lysis buffer warm to room temperature
8. Add 2 mls 1x lysis buffer to each tube. Pipette up and down to mix.
9. Wrap rack with tinfoil and incubate on shaker for 25 minutes at room temperature
10. Centrifuge at 300 x g for 5 minutes
11. Decant supernatant
12. Add 2 mls of cold Stain Buffer to each tube. Pipette up and down to mix.
13. Centrifuge at 300 x g for 5 minutes
14. Decant supernatant
15. Prepare 2% formaldehyde solution: Combine 0.8 mls 20% formaldehyde with 7.2 mls cold Stain Buffer. (Note: 2% formaldehyde should not be made more than 30 minutes in advance and should be discarded immediately after use. It cannot be saved for future use)
16. Add 200 µl of 2% formaldehyde to each tube. Pipette up and down to mix.
17. Replace caps on tubes; they should snap firmly into place (should hear two clicks)
18. Wrap rack in a new sheet of tinfoil
19. Store at 4°C until ready to be shipped
20. Ship sample to Immunophenotyping Core using the procedures described in Section 6.2

3.5.6 Tongue scraping and Oral rinse

Prior to anesthesia:

Step 1: Tongue Scraping

1) Using moderate pressure, rapidly scrap the tongue 6 times to accumulate a buildup of debris on the tongue scraper.
2) Swirl the tongue scraper in the 90 mL ORAL specimen container with 20 mL of RNALater to remove tongue debris. Discard tongue scraper.
3) Repeat steps 1 and 2, using a new tongue scraper, 2 more times.

4) Please keep lid of specimen container closed as much as possible to reduce contamination.

The purpose of the Tongue Scraping is to collect bacterial communities on the tongue and clean the mouth to decrease contamination of the bronchoscope.

Step 2: Oral Rinse

1) Have subject cough 5 times and then ACTIVELY gargle 10 cc of sterile saline for 20 seconds.
    a. Use a stopwatch to time the gargle. Do not include any time if patient pauses to rest.
    b. Please record time that patient gargled on collection form.
2) At end of gargle, instruct patient to swish the saline in the mouth for 5 seconds.
3) Patient will spit oral rinse into the ORAL specimen container, gently spitting into the RNALater.
4) Gently invert RNALater 10 times to thoroughly mix.
5) Transfer into 3x15 ml conical for better storage at GIC
6) Place on ice or store at 4 degrees C overnight.
7) Move samples to -80 degrees C freezer until ready to ship.

The purpose of the Oral Rinse is collect samples of the bacterial communities in the oral mouth and upper airway and to remove loose debris that may contaminate airway sample.

**** **PLEASE NOTE:** If there is an unforeseen delay from the time of ORAL collection and bronchoscopy and the elapsed time exceeds greater than 60 minutes, please tongue scrape patients 1x and swish with sterile saline solution 1x prior to bronchoscopy. If a second oral rinse is completed do not save the saline or tongue scrapping from this rinse. Write a note on the collection sheet noting that a second oral rinse was completed.

3.5.7 Upper Airway Anesthesia

As the details of the topical anesthesia are unlikely to affect the samples, this aspect of the procedure is left to the discretion of the investigator and bronchoscopist. The spirit of this guideline is to minimize the lidocaine dose delivered to the subject. For purposes of calculation, all lidocaine administered is to be recorded, whether delivery is via gargle, aerosol, spray, or instillation. **For subject safety, the following limit on lidocaine dose**

is suggested, based on a publication (Langmack EL, et al. Chest 2000; 117: 1055-60) which demonstrates levels after administration of up to 9 mg/kg of lidocaine: 600 mg or 9 mg/kg whichever is less.

3.5.8 Pre-Bronchoscopy Medication (administered by RN or MD)

Sedatives/anxiolytics: Fentanyl and Versed given in small incremental doses as needed (local institutional guidelines for conscious sedation must be followed). If sedation is administered, have Narcan and Romazicon at the ready. Premedication with Benadryl is allowed at the discretion of the bronchoscopist.

3.5.9 Vital Sign Monitoring

Record blood pressure, oxygen saturation, and heart rate before and after administration of the pre-bronchoscopy medication. Monitor and record blood pressure, oxygen saturation, pulse rate and oxygen flow rate every 2-4 minutes during bronchoscopy. Inform the physician if there is a fall in oxygen saturation, a change in blood pressure, and significant change in heart rate, mental status, or level of pain.

3.5.10 Saline alone and Suctioning saline through the scope for microbiome saline control samples

1. Draw 2.5ml of 0.9% sterile saline into a sterile 20cc syringe (use the saline that will be used for BAL). Transfer this 2.5mL of saline into a 15mL conical tube which contains 10mL of RNAlater and which is labelled “SALINE ALONE”. Place in a 4 degrees C refrigerator for 24 hours, then store at -80^{0}C.
2. Draw another 5.5 ml of 0.9% sterile saline into the sterile 20cc syringe (use the saline that will be used for BAL). Flush these 2.5ml of saline through the bronchoscope and collect in a sterile specimen cup prior to start of bronchoscopy.
3. Transfer this 2.5mL of flushed saline into a second 15mL conical tube which contains 10mL of RNAlater and which is labelled “SALINE THROUGH SCOPE”. Place in a 4 degrees C refrigerator for 24 hours, then store at -80^{0}C.

3.5.11. Introducing the Bronchoscope into the Airways

Attach the pulse oximeter, automatic sphygmomanometer, and nasal cannulae to the volunteer. Activate both monitors and set the oxygen flow between 0.5 - 4 Lpm as clinically indicated. Activate the primary suction. Set up the secondary suction system for aspirating secretions from the pharynx.

Depending upon the preference of the bronchoscopist, have the volunteer sit on the side of the gurney or lie supine. Place the bite block in position. Squeeze approximately 1 tablespoon of 2% lidocaine jelly onto a 4” X 4” sponge gauge. Wipe the distal region of the bronchoscope beginning approximately 1/2 inch proximal from the tip of the bronchoscope and continuing proximal for approximately 4-5 inches.

3.5.12 Lower Airway Anesthesia

Lower airway anesthesia with lidocaine should be performed using local protocols, with the following limit on lidocaine dose: 600 mg or 9 mg/kg whichever is less.
Alert the bronchoscopist when 300 mg of lidocaine has been delivered

3.5.13 Airway Inspection

After bronchoscope passes vocal cords, bronchoscopist should inspect the airways first prior to sample collection. Begin inspection in distal segments while numbing with lidocaine as described above in step 3.10. Grading of airway appearance will be recorded after the procedure using the "Bronchitis index" (4). If abnormal findings are noted, the bronchoscopist has the option of terminating the procedure and providing appropriate follow-up per physician judgment.

4 BRONCHOSCOPIC SAMPLE COLLECTION AND PROCESSING PROCEDURES

The order of bronchoscopic collections may be revised upon the judgment of the investigator performing the procedure.

4.0 Overview

The preferred order and locations of procedures are as follows

1. Protected brush specimen(s) in a lower lobe bronchus (3 brushes total in most instances)
2. Wash/BAL (approach: 20cc x1 [repeat if no return]/then 2x40cc, 1x50cc into one segment), manual suction
3. Repeat wash/BAL in another segment
4. Cytological brushes x 3 in ipsilateral lower lobe bronchi
5. Optional small airway brushings
6. Endobronchial biopsies in the contralateral lung (up to 8 adequate biopsies, beginning in lower lobe and proceeding cranially)

4.1 Protected brush specimen

Three protected brush specimens will be performed in a lower lobe bronchus during most bronchoscopies.

Brush type: Disposable Microbiology Brushes (ConMed, Catalogue # 130, phone # (800) 448-6506).

Advance the Disposable Microbiology Brush into the segment and "drop" the plug. Advance the brush and gently brush the bronchial mucosa (about 0.5 inch) while rotating

the brush 360 degrees. After about 7 seconds of brushing, retract the brush completely into the inner catheter. Then retract the inner catheter into the outer catheter by pulling the blue and white section apart. Withdraw the microbiology brush assembly from the bronchoscope. See description below on how to remove the brush. Collect 2 additional microbiology brush samples.

Microbiology brush handling: (n=3)

1) Remove each brush sample by first wiping the outer catheter approximately 5 mm distal to the inner catheter with alcohol prep (proposed cut site). Then cut the outer catheter at the alcohol cleansed site, and discard.

2) Then, completely advance the inner catheter. Wipe the inner catheter about 5 mm distal to the brush tip (proposed cut site) with alcohol prep, and then cut the inner catheter at the alcohol cleansed site.

3) Advance the brush directly and completely into the 2.0 mL microtube containing RNALater™. Cut the wire level with the top of the tube, so that brush is kept fully immersed in the RNALater™. Screw the cap on tightly and place on ice.

4) Repeat with new tube for each brush.

5) Place at 4 degrees C refrigerator for 24 hours, then transfer to -80 degrees C freezer.

6) Ship on dry ice to the BSP following the shipping procedures outlined in Section 6.1.

   N.B. - After processing using the Qiagen AllPrep DNA/RNA kit, the expected yield will be 1mcg of DNA on average. For PhyloChip analyses one can start with 30ng of DNA template for each of 12 different PCR reactions (run on a range of gradients). Thus, 360ng represents a minimum necessary yield per sample (not accounting for reruns or validation experiments).

4.2 Initial airway wash

Two separate 20cc washes will be performed (the first aliquot of each BAL maneuver) and then pooled. These washes will be done using sterile saline ipsilateral to the initial brush site (RML or lingula) and from two distinct segments. Then, after each wash, BAL is continued in the same segment (see 4.4). If the bronchoscopist cannot wedge more distal than RML or lingual (happens ~10% of the time), then upper lobe anterior segment is next choice. A 10cc air chaser is introduced behind the wash sample and manual suction is used. Each 20cc aliquot can be repeated a second time if there is no return. The sample is collected into specifically designated specimen traps (lidocaine free) on ice.

First, pool the washes from the two airway wash maneuvers.

Option 1:

1. **If ≥8mL is returned, set aside 4.5 mL of bronchial wash for microbiome studies and process the rest for total cell count, differential, storage of supernatants and RNA prep of cell pellet as described in steps below.**
2. Microbiome:
    a. Put 4.5 mL of bronchial wash into a 50 mL sterile conical tube containing 9 mL RNAlater, mix gently
    b. Place in a 4 degrees C refrigerator for 24 hours, then transfer to one 15ml conical and store in -80 degrees C freezer (for microbiome studies).
    c. Ship the frozen 15mL tube to the BSP following the shipping procedures outlined in Section 6.1

3. Total cell count:
    a. Take 10µl of remaining Wash into an Aliquot tube
    b. Add an equal volume of Turks solution
    c. Pipette 10 µl of the mixed Wash fluid/Turks solution onto one side of the hemocytometer chamber and cover with the cover slip
    d. Count all non-red blood cells, i.e. epithelial and white cells including monocytes and macrophages, in the 4 large corner squares.
    e. Determine cell count: (Cells overlying the lines to the top and left will be counted once and cells overlying lines on the bottom and right should NOT be counted).

    Cell Count:

    # cells/mL = (# cells in 4 large squares/4) x 10^4 x 2 (to account for 1:1 dilution with Turks solution)

    Total # cells: = # cells/mL x total volume (mL) of Wash

Record this information on the Bronchoscopy Specimen Processing (BPW) form.

4. Transfer remaining wash into two 15 mL polypropylene conical tubes (equal volume).
5. Centrifuge at 300 x g for 5 min.
6. Decant the supernatants from two 15 mL tubes and save for up to 40 500µl aliquot samples.
7. Store aliquots at -80 degrees Celsius
8. Ship created in step 6 to the BSP following the shipping procedures outlined in Section 6.1

9. Cell Differential using one of the two 15mL tubes: Determine the volume of normal PBS to prepare cell suspension that will yield approximately 40,000-50,000 cells per cytocentrifuge slide:

   Total # cells in the wash = # cells/mL x mL of wash in one of the tubes

   Volume of normal PBS to add (mL) = # cells in the tube / 0.7 x 10^6

   For example:
   If the # cells in the tube = 0.2 x 10^6/mL x 10 mL = 2.0 x 10^6

   Then the volume of normal PBS (mL) to be added = (2.0 x 10^6)/ (0.7 x 10^6) = 2.85 mL

   Add the volume of normal PBS calculated from step 3 to the 15 mL conical tube (with cell pellet at the bottom) and mix well with a pipette carefully. Avoid creating bubbles (do not vortex)
   Transfer 60 all of the cell suspension into Shandon cytospin funnels and centrifuge at 500 rpm for 5 min (to make up to 2 separate slides if possible).
   Check cell density under microscope before staining. If necessary adjust dilution of the cell suspension fluid.

Stain two Wash cytospin slides with the Shandon Diff-quick kit:

i) Dip in fixative for about 10 seconds. Drain/blot excess fixative from end of slide
ii) Dip in Solution I for 5 seconds. Drain/blot excess solution.
iii) Dip in Solution II for 15 to 20 seconds. Drain excess solution. (Dipping in Solution II for a longer period of time will heighten the contrast between blue cytoplasm and purple nucleus.)
iv) Rinse with distilled water. Allow water to gently run down slide, avoid disturbing cell layer
v) Check slide for staining quality under microscope. Re-stain if necessary, repeating steps 2 - 4.
vi) Allow to air dry, apply ~2 - 3 drops of Cytoseal 60 adjacent to cell layer. Place coverslip on the slide at a 90 degree angle and gently lower to slide surface. Squeeze out bubbles. Allow to dry before placing inside mailers.
vii) Ship to BSP using the procedures described in Section 6.1

10. RNA prep of cell pellet using the other 15 mL tube:
    a. Resuspend cell pellet resuspend in 600µl QIAzol.
    b. Transfer resuspended pellet to a 1.5 ml O-ring aliquot and vortex for 1 min on highest setting
    c. Store at -70 or -80 degrees C
    d. Ship to the BSP following the shipping procedures outlined in Section 6.1

Option 2.

1) **If <8mL is returned, process for total cell count, differential, storage of supernatants and RNA prep of cell pellet as described in steps below.**
2) Total cell count:
   a) Take 10µl of remaining Wash into an Aliquot tube
   b) Add an equal volume of Turks solution
   c) Pipette 10 µl of the mixed Wash fluid/Turks solution onto one side of the hemocytometer chamber and cover with the cover slip
   d) Count all non-red blood cells, i.e. epithelial and white cells including monocytes and macrophages, in the 4 large corner squares.
   e) Determine cell count: (Cells overlying the lines to the top and left will be counted once and cells overlying lines on the bottom and right should NOT be counted).

Cell Count:

cells/mL = (# cells in 4 large squares/4) x 10^4 x 2 (to account for 1:1 dilution with Turks solution)

Total # cells: = # cells/mL x total volume (mL) of Wash

Record this information on the Bronchoscopy Specimen Processing (BPW) form.

3) Transfer remaining wash into two 15 mL polypropylene conical tubes (equal volume).
4) Centrifuge at 300 x g for 5 min.
5) Decant the supernatants from the 15 mL tubes and save for up to 40 500µl aliquot samples.
6) Store aliquots at -80 degrees Celsius
7) Ship created in step 6 to the BSP using the procedures described in Section 6.1.

8) Cell Differential using one of the two 15mL tubes: Determine the volume of normal PBS to prepare cell suspension that will yield approximately 40,000-50,000 cells per cytocentrifuge slide:

   Total # cells in the wash = # cells/mL x mL of wash in one of the tubes

   Volume of normal PBS to add (mL) = # cells in the tube / 0.7 x 10^6

   For example:
   If the # cells in the tube = 0.2 x 10^6/mL x 10 mL = 2.0 x 10^6

Then the volume of normal PBS (mL) to be added = $(2.0 \times 10^6)/ (0.7 \times 10^6) =$ 2.85 mL

Add the volume of normal PBS calculated from step 3 to the 15 mL conical tube (with cell pellet at the bottom) and mix well with a pipette carefully. Avoid creating bubbles (do not vortex)

Transfer 60 µl of the cell suspension into Shandon cytospin funnels and centrifuge at 500 rpm for 5 min (to make 2 separate slides if possible).

Check cell density under microscope before staining. If necessary adjust dilution of the cell suspension fluid.

Stain two Wash cytospin slides with the Shandon Diff-quick kit:

i) Dip in fixative for about 10 seconds. Drain/blot excess fixative from end of slide
ii) Dip in Solution I for 5 seconds. Drain/blot excess solution.
iii) Dip in Solution II for 15 to 20 seconds. Drain excess solution. (Dipping in Solution II for a longer period of time will heighten the contrast between blue cytoplasm and purple nucleus.)
iv) Rinse with distilled water. Allow water to gently run down slide, avoid disturbing cell layer
v) Check slide for staining quality under microscope. Re-stain if necessary, repeating steps 2 - 4.
vi) Allow to air dry, apply ~2 - 3 drops of Cytoseal 60 adjacent to cell layer. Place coverslip on the slide at a 90 degree angle and gently lower to slide surface. Squeeze out bubbles. Allow to dry before placing inside mailers.
vii) Ship to BSP using the procedures described in Section 6.1

9) <u>RNA prep of cell pellet using the other 15 mL tube</u>:
   a. Resuspend cell pellet resuspend in 600µl QIAzol.
   b. Transfer resuspended pellet to a 1.5 ml O-ring aliquot and vortex for 1 min on highest setting
   c. Store at -70 or -80 degrees C
   d. Ship to the BSP using the procedures outlined in Section 6.1.

4.3 Bronchoalveolar lavage

While remaining in the same segment as the airway wash, bronchoalveolar lavage (BAL) is performed by instilling sterile saline in the following volumes: 2x40cc then 1x50cc. If <15cc of fluid is returned from the combined volume of the 20cc wash and the 2x40cc of lavage, then the 1x50cc lavage should not be performed. After the first wash/BAL is completed, then another segment is cannulated and the wash and BAL are repeated. Specifically, 20cc for wash (with an optional second 20cc wash aliquot if no return the first time), and 2x40cc and 1x50cc for lavage is instilled into this second segment Again, if <15cc of fluid is returned from the combined volume of the 20cc wash and the 2x40cc

of lavage, then the 1x50cc lavage should not be performed. The BAL return from both segments is collected into specifically designated specimen traps (lidocaine free) on ice. During the BAL procedure, a 10cc air chaser is introduced behind the lavage sample and manual suction is used. Return specimen traps may need to be switched when full, and are collected on ice. It is important to switch back to waste trap and wall suction before bronchoscopist breaks seal, in order to enrich epithelial cell return. If subject has difficulty with coughing, abort the BAL and continue on to brushings and biopsies. If the seal was broken prematurely (i.e. if subject coughed hard enough), do not return to segment. Instead, proceed with second round of wash and lavage in new segment.

4.3.1 Initial BAL Handling

1. Do not filter the lavage fluid
2. Record total volume of return fluid
3. Mix pooled BAL fluid in either a sterilized beaker or in multiple 50 mL conical tubes (swirling gently)
4. Put 2ml of this pooled lavage fluid in a 15mL sterile container and freeze immediately at -80C
4. Store BAL fluid on ice throughout processing

4.3.2 BAL cell count

1. Aliquot 10 µl of the fluid into an Aliquot tube
2 Add an equal volume of Turks solution.
3. Pipette 10 µl of the mixed BAL fluid/Turks solution onto one side of the hemocytometer chamber and cover with the cover slip.
4. Count all non-red blood cells, i.e. epithelial and white cells including monocytes and macrophages, in the 4 large corner squares.
5. Determine cell count: (Cells overlying the lines to the top and left will be counted once and cells overlying lines on the bottom and right should NOT be counted).

   Cell Count:

   # cells/mL = (# cells in 4 large squares/4) x 10^4 x 2 (to account for 1:1 dilution with Turks solution)

   Total # cells: = # cells/mL x total volume (mL) of BAL fluid

6. Record the number of cells per milliliter (# cells /mL)

   For example:

   If you have 100 mL BAL fluid and the number of cells on the 4 large squares is 80:

   Then: # cells/mL= (80/4) x 10^4 x 2 = 0.4 x 10^6 cells/mL

Total # cells: = 0.4 x 10^6 cells/mL x 100 mL= 40 x 10^6

Record this information on the Bronchoscopy Specimen Processing (BPW) form.

4.3.3 BAL cell and supernatant handling

1. Microbiome:
    a. Put 4.5 mL of previously pooled/mixed BAL fluid into a 50 mL sterile conical tube containing 9 mL RNAlater, mix gently
    b. Place at 4 degrees C refrigerator for 24 hours
    c. Transfer to one 15ml conical and store in -80 degrees C freezer (for microbiome studies).
    d. Ship to BSP using the procedures outlined in Section 6.1

2. Transfer 10 mL of previously pooled/mixed BAL fluid into one 15 mL polypropylene conical tube for cell count (further details below).
3. Transfer 2ml of previously pooled/mixed BAL fluid into a sterile 15mL conical tube for future microbial culture analyses. Store in -80 degrees C freezer for later shipping to the BSP using the procedures outlined in Section 6.1.
4. Transfer the remaining fluid approximately equally across two 50 mL tubes (or three+ 50 mL tubes if the BAL volume exceeds 100mL).
5. Record the number of ml of remaining BAL volume left over after aliquoting steps 1 and 2 above.
6. Centrifuge this remaining BAL fluid at 300 x g for 5 min.
7. Decant the supernatant from 15 mL tube and save for aliquot samples (after combining with supernatant from step 6 below). Reserve cell pellet from 15 mL tube for production of cytospin slides (see below).
8. Decant the supernatant from the 50 mL tube(s) and save for additional aliquot samples. Make 40 x 1mL aliquots and 4 x 15mL tubes of supernatant. Any additional BAL supernatant should discarded or, if prior approval has been obtained, be stored at the site. Reserve cell pellet from 50 mL tube(s) for Flow cytometry and alveolar macrophage isolation as described below (#8 and Sections 4.4.6 and 4.4.7).
9. Store aliquots made in steps 4 and 5 at -80 degrees Celsius
10. Aliquots created in steps 4 and 5 are shipped to the BSP using the procedures described in Section 6.1.
11. Media used for the next step should be made in advance as follows: The RPMI will be shipped to each center by the GIC. The RPMI should have an expiration that is dated June 2014 or later. Bottles of RPMI should be stored at 4 degrees and protected from light (e.g. cover with foil) for the duration of the study. The aliquots of frozen FBS will be 1.1 ml in volume and shipped to each center from UCSF. Store the aliquots protected from light at -20 degrees C. To make the media, add 49 ml of RPMI to a 50 ml sterile conical tube. Thaw one aliquot of FBS (each aliquot will contain1.1ml) in a 37 degrees C water bath. As soon as the FBS is thawed, remove from water bath and transfer 1 ml to the conical tube. Sterile filter the media using the 0.22 um sterile filters attached to sterile 50 ml conical tube (Catalogue # SCGP00525

Millipore Steriflip Sterile Disposable Vacuum Filter Units). Label and date the conical tube. Store at 4 degrees C and protect from light (e.g. with foil). This media may be used for the macrophage isolations for a total time period of one month. If the media is not used within a month, discard any remaining media and repeat steps to make a new batch of macrophage isolation media.

12. Gently resuspend cell pellet in the first 50ml conical with 10ml of media made in step 8 above (RPMI and 2% FBS) to bring the cells into suspension using a serological pipette Transfer this 10ml aliquot into the next 50 ml conical and resuspend that pellet as well. Do this with the third 50 ml tube if there is one.
13. To determine the cell count of this new aliquot, multiply the volume recorded in step 3 above with the number of cells/ml recorded in section 4.3.2, step 6. This will be the number of total cells present in the new aliquot. Take this number and divide it by 10 ml. This is the new concentration of cells/ml.
14. Calculate the volume of the 10ml aliquot which contains ~1,000,000 cells. This will be transferred into a 15ml conical tube for alveolar macrophage plating (see section 4.4.6). STORE ON ICE until ready for processing. (Example: (X ml aliquot) * (1,000,000 cells/ml) = 1,000,000 cells. Solve for X which would be 1 ml).
15. Allot the remaining media/cells into another 15ml conical tube for immunophenotyping (see Immunophenotyping protocol, section 4.4.7). Ideally, this aliquot would contain >$8x10^6$ cells.

4.3.4 BAL cell cytospin preparation

2) Use the cell pellet reserved from the 15 mL conical tube (Section 4.3.3 step 2 which contained 10 mL of BAL fluid) to prepare cytospins.
3) Label Frost Plus slides with the GIC provided sample label.
4) Determine the volume of normal PBS to prepare cell suspension that will yield approximately 40,000-50,000 cells per cytocentrifuge slide:

   Total # cells in the 10 mL tube = # cells/mL x 10mL

   Volume of normal PBS to add (mL) = # cells in the 10 mL tube / 0.7 x 10^6

   For example:
   If the # cells in the 10 mL tube = 0.2 x 10^6/mL x 10 mL = 2.0 x 10^6

   Then the volume of normal PBS (mL) to be added = (2.0 x 10^6)/ (0.7 x 10^6) = 2.85 mL

5) Add the volume of normal PBS calculated from step 3 to the 15 mL conical tube (with cell pellet at the bottom) and mix well with a pipette carefully. Avoid creating bubbles (do not vortex).
6) Transfer 60 µl of the cell suspension into each of four Shandon cytospin funnels and centrifuge at 500 rpm for 5 min (to make 4 separate slides).
7) Check cell density under microscope before staining. If necessary adjust dilution of the cell suspension fluid.

8) Stain two slides and fix two slides with the Shandon Diff-quick kit:
   a) To stain two BAL cytospin slides:
      i) Dip in fixative for about 10 seconds. Drain/blot excess fixative from end of slide
      ii) Dip in Solution I for 5 seconds. Drain/blot excess solution.
      iii) Dip in Solution II for 15 to 20 seconds. Drain excess solution. (Dipping in Solution II for a longer period of time will heighten the contrast between blue cytoplasm and purple nucleus.)
      iv) Rinse with distilled water. Allow water to gently run down slide, avoid disturbing cell layer
      v) Check slide for staining quality under microscope. Re-stain if necessary, repeating steps 2 - 4.
      vi) Allow to air dry, apply ~2 - 3 drops of Cytoseal 60 adjacent to cell layer. Place coverslip on the slide at a 90 degree angle and gently lower to slide surface. Squeeze out bubbles. Allow to dry before placing inside mailers.
      vii) Ship to BSP using the procedures described in Section 6.1
   b) To fix remaining BAL cytospin slides:
      i) Dip in fixative solution for about 10 seconds. Drain/blot excess fixative from end of slide.
      ii) Allow to air-dry before inserting into slide shippers

4.3.5 Staining cytospin slides

Stain and coverslip two slides fully. For any remaining slides (2 for BAL) perform fixation step only (Step 1– do not coverslip, or stain). Allow fixed slides to air-dry before inserting into slide shippers.

Use Shandon Diff-quick kit:

1. Dip in fixative solution for about 10 seconds. Drain/blot excess fixative from end of slide.
2. Dip in Solution I for 5 seconds. Drain/blot excess solution.
3. Dip in Solution II for 15 to 20 seconds. Drain excess solution. (Dipping in Solution II for a longer period of time will heighten the contrast between blue cytoplasm and purple nucleus.)
4. Rinse with distilled water. Allow water to gently run down slide, avoid disturbing cell layer
5. Check slide for staining quality under microscope. Re-stain if necessary, repeating steps 2 - 4.
6. Allow to air dry, apply ~2 - 3 drops of Cytoseal 60 adjacent to cell layer. Place coverslip on the slide at a 90 degree angle and gently lower to slide surface. Squeeze out bubbles. Allow to dry before placing inside mailers.
7. Ship to BSP using the procedures described in Section 6.1

4.3.6 Alveolar macrophage isolation

1. BALF will be processed as per SPIROMICS protocol (see item 9 under 4.3.3)
2. One million alveolar macrophages will be aliquoted in media as described in section 4.3.3. and put on ICE.
3. All participating centers will begin processing of macrophages **exactly 2 hours** after they are collected. **Note in DMS if the macrophage processing started earlier or later than 2 hours after collection**. This should be done in the Tissue Culture Hood and should be done with sterile technique to prevent cell contamination.

4. Macrophage Processing Cells: The aliquot used for this section will come from section 4.3.3. Media (RPMI + 2% FBS) will be made as per section 4.3.3 step 9.
    1. Bring up the volume of the 1,000,000 BAL cells to 5 ml using media. Gently resuspend cells by pipetting up and down ~10 times at slow speed
    2. Aliquot cell suspension onto a tissue culture treated plate (60 mm Fisher scientific cat # 430589) and replace the plate lid over plate after aliquoting cells.
    3. Incubate covered plate for 30 min at 37 degrees C, 5% CO_2
    4. At end of incubation, remove the plate cover and slightly tilt plate and aspirate off media (discard media)---move to next step quickly to avoid cell drying
    5. Then, gently aliquot 5 ml PBS/Ca + Mg (vendor: Fisher Scientific, Cat#: MT-21-030-CV) to the corner of plate; gently swirl plate in 3 rotations and tilt plate again and aspirate PBS
    6. Repeat step #5
        a. Move quickly between steps so that cells don't dry
    7. Then add 600 µl QIAzol buffer and make sure the buffer coats the entire surface of plate.
    8. Incubate covered plate for 5 min at room temperature
    9. Then using RNAse-free pipette tips, pipette up and down approximately 5-10 times to lyse all adhered macrophages
    10. Using pipette, transfer the lysed macrophages in QIAzol buffer into an RNAse free tube (1.5 ml O-ring aliquot)
    11. Label tube
    12. Immediately transfer tube to -80 deg freezer for storage
    13. Ship to BSP using the procedures described in Section 6.1

4.3.7 Immunophenotyping BAL protocol

1. Use the 15 ml conical tube set aside for immunophenotyping (in RPMI) (see item 10 under 4.4.2)
2. Centrifuge at 300 x g for 5 minutes
3. Decant supernatant and resuspend in 1.5 mLs cold Stain Buffer
4. Briefly centrifuge immunophenotyping BAL assay antibody tubes (supplied by immunophenotyping core) at 300 x g for 2 minutes and uncap tubes
5. Add 100 µl of BAL sample to each tube. Gently mix tube by swirling.
6. Wrap rack with tinfoil to protect from light

7. Incubate on shaker for 25 minutes at room temperature
8. Add 2 mls of cold Stain Buffer to each tube. Pipette up and down to mix.
9. Centrifuge at 300 x g for 5 minutes
10. Decant supernatant
11. Prepare 2% formaldehyde solution: Combine 0.3 mLs 20% formaldehyde with 2.7 mLs Stain Buffer. (Note: 2% formaldehyde should not be made more than 30 minutes in advance and should be discarded immediately after use. It cannot be saved for future use)
12. Add 200 µl of 2% formaldehyde to each tube. Pipette up and down to mix
13. Replace caps on tubes; they should snap firmly into place (should hear two clicks)
14. Wrap rack in a new sheet of tinfoil
15. Store at 4°C until ready to be shipped
16. Ship sample to Immunophenotyping Core using the procedures described in Section 6.2

4.4 EPITHELIAL BRUSHINGS

4.4.1 Required large airway cytological brushings: Up to six separate airway epithelial brushings are obtained from lower-lobe segments (3 with protected specimen brushes for microbiome analyses [handling of these described in detail above section 4.2] and 3 with disposable cytology brushes for airway epithelial cells [ConMed, Catalog # 149, 3mm diameter x 11mm long, phone # (800) 448-6506]).

For cytological brushings, deploy each brush at segmental or sub-segmental carina. Advance 1-2 cm but keep the brush in sight. GENTLY brush the side-wall avoiding any bleeding, rotating the brush, and brushing back and forth approximately 5 times. If a brush has blood on it, dispose of the brush and flush the bronchoscopic channel with a small volume (2-5 mL) of sterile saline before proceeding with additional brushes.

Cytological brush handling (n=3)

1. Remove each brush by wiping the outer catheter approximately 5 mm distal the brush tip with an alcohol prep (proposed cut site). Then cut the outer catheter at the alcohol cleansed site, and discard.
2. Place each brush in a 2 mL screw cap tube containing 1mL RNase-Free PBS on ice. Cut the wire and close the cap over the brush.
3. Store brushing tubes on ice until further processing.
4. After the bronchoscopy, vortex each tube at setting 3-4 (low speed such that only a small amount of foam results) for ~ 3 minutes.
5. Remove brush and place in clean 2 mL tube until cell counts performed.
6. Pool cells (in suspension) from the 3 brushings into one 15 mL polypropylene conical tube.
7. Store on ice until finished processing.

8. Set aside a small volume (200 µl from the first 15 mL conical tube for total and differential cell counts)

    a. Total cell count:
        i. Take 10µl of Epithelial brush sample into an Aliquot tube
        ii. Add an equal volume of Turks solution
        iii. Pipette 10 µl of the mixed brush fluid/Turks solution onto one side of the hemocytometer chamber and cover with the cover slip
        iv. Count all non-red blood cells, i.e. epithelial and white cells including monocytes and macrophages, in the 4 large corner squares.
        v. Determine cell count: (Cells overlying the lines to the top and left will be counted once and cells overlying lines on the bottom and right should NOT be counted).

            Cell Count:

            # cells/mL = (# cells in 4 large squares/4) x 10^4 x 2 (to account for 1:1 dilution with Turks solution)

            Total # cells: = # cells/mL x total volume (mL) of Wash

        Record this information on the Bronchoscopy Specimen Processing (BPW) form.

    b. The remaining volume should be used to prepare 2 cytospin, fixed and stained slides (60 µl each) as described above in step 4.3.5: "Staining Cytospin slides."
        i. Stain two Epithelial Brush cytospin slides with the Shandon Diff-quick kit:
            1. Dip in fixative for about 10 seconds. Drain/blot excess fixative from end of slide
            2. Dip in Solution I for 5 seconds. Drain/blot excess solution.
            3. Dip in Solution II for 15 to 20 seconds. Drain excess solution. (Dipping in Solution II for a longer period of time will heighten the contrast between blue cytoplasm and purple nucleus.)
            4. Rinse with distilled water. Allow water to gently run down slide, avoid disturbing cell layer
            5. Check slide for staining quality under microscope. Re-stain if necessary, repeating steps 2 - 4.
            6. Allow to air dry, apply ~2 - 3 drops of Cytoseal 60 adjacent to cell layer. Place coverslip on the slide at a 90 degree angle and gently lower to slide surface. Squeeze out bubbles. Allow to dry before placing inside mailers.

7. Ship to BSP using the procedures described in Section 6.1.These cytospins should be sent to the GIC for cell differentials (clearly labeled and distinct from the BAL samples).

Remaining volume of cell solution in the 15 mL conical tube will be used for initial steps in RNA preparation (by the sites as described below).

Initial steps in Epithelial RNA preparation (to be done at the Clinical Centers):

1. Use cell suspension from the 3 brushings previously transferred to a 15 mL conical tube
2. Centrifuge at 300 X g for 5 minutes
3. Discard supernatant
4. Disrupt the cells by adding 600 µl QIAzol Lysis Reagent. For pelleted cells, loosen the cell pellet thoroughly by flicking the tube.
5. Vortex for 1 minute on highest setting to lyse cells. Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.
6. Transfer suspension to 1.5 mL Aliquot tube (with O-ring provided)
7. Ship to the BSP using the procedures described in Section 6.1

Homogenized cell lysates in QIAzol Lysis Reagent can be stored at -70 degrees C for several months.

4.4.2. Optional Small Airway Cytological Brushings

Up to 12 separate airway epithelial brushings may be obtained from distal airways if IRB approval at your site has been obtained for this sample. A Kimberly-Clark cytology brush 2.0 mm diameter, reference #60311 (different from the large airway cytology brush) is used.

For distal cytological brushings, deploy each brush at the orifice of a right and/or left lower lobe subsegmental bronchus (anterior, lateral and posterior basal, but not superior or medial) and advance carefully 3-5 cm, 1 cm at a time, stopping if any resistance is encountered and withdrawing and repositioning the sheath if necessary. After extending the sheath a maximum of 5 cm, the brush is advanced out of the sheath. If any resistance is encountered the sheath may be pulled back as the brush is advanced. With the brush fully deployed the sheath is very gently moved in a proximal/distal repetition for 15-20 strokes for each brushing. The brush is withdrawn into the sheath and then the sheath is removed from the scope.

<u>Optional Distal Cytological brush handling</u>

1. Remove each brush by wiping the outer catheter approximately 5 mm distal the brush tip with an alcohol prep (proposed cut site). Then cut the outer catheter at the alcohol cleansed site, and discard.
2. Place each brush in a 2 mL screw cap tube containing 1mL RNase-Free PBS on ice. Cut the wire and close the cap over the brush.
3. Store brushing tubes on ice until further processing.
4. After the bronchoscopy, vortex each tube at setting 3-4 (low speed such that only a small amount of foam results) for ~ 3 minutes.
5. Remove brush and place in clean 2 mL tube until cell counts performed.
6. Pool cells (in suspension) from the 3 brushings into one 15 mL polypropylene conical tube.
7. Store on ice until finished processing.
8. Set aside a small volume (200 µl from the first 15 mL conical tube for total and differential cell counts)
    a. Total cell count:
        i. Take 10µl of Epithelial brush sample into an Aliquot tube
        ii. Add an equal volume of Turks solution
        iii. Pipette 10 µl of the mixed brush fluid/Turks solution onto one side of the hemocytometer chamber and cover with the cover slip
        vi. Count all non-red blood cells, i.e. epithelial and white cells including monocytes and macrophages, in the 4 large corner squares.
        vii. Determine cell count: (Cells overlying the lines to the top and left will be counted once and cells overlying lines on the bottom and right should NOT be counted).

    Cell Count:

    # cells/mL = (# cells in 4 large squares/4) x 104 x 2 (to account for 1:1 dilution with Turks solution)

    Total # cells: = # cells/mL x total volume (mL) of Wash

    Record this information on the Bronchoscopy Specimen Processing (BPW) form.

    b. The remaining volume should be used to prepare 2 cytospin, fixed and stained slides (60 µl each) as described above in step 4.3.5: "Staining Cytospin slides."

        ii. Stain two Epithelial Brush cytospin slides with the Shandon Diff-quick kit:
            1. Dip in fixative for about 10 seconds. Drain/blot excess fixative from end of slide

2. Dip in Solution I for 5 seconds. Drain/blot excess solution.
3. Dip in Solution II for 15 to 20 seconds. Drain excess solution. (Dipping in Solution II for a longer period of time will heighten the contrast between blue cytoplasm and purple nucleus.)
4. Rinse with distilled water. Allow water to gently run down slide, avoid disturbing cell layer
5. Check slide for staining quality under microscope. Re-stain if necessary, repeating steps 2 - 4.
6. Allow to air dry, apply ~2 - 3 drops of Cytoseal 60 adjacent to cell layer. Place coverslip on the slide at a 90 degree angle and gently lower to slide surface. Squeeze out bubbles. Allow to dry before placing inside mailers.
7. Ship to BSP using the procedures described in Section 6.1 These cytospins should be sent to the GIC for cell differentials (clearly labeled and distinct from the BAL samples).

Remaining volume of cell solution in the 15 mL conical tube will be used for initial steps in RNA preparation (by the sites as described below).

Initial steps in Epithelial RNA preparation (to be done at the Clinical Centers):

1. Use cell suspension from the 3 brushings previously transferred to a 15 mL conical tube
2. Centrifuge at 300 X g for 5 minutes
3. Discard supernatant
4. Disrupt the cells by adding 600 µl QIAzol Lysis Reagent. For pelleted cells, loosen the cell pellet thoroughly by flicking the tube.
5. Vortex for 1 minute on highest setting to lyse cells. Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.
6. Transfer suspension to 1.5 mL Aliquot tube (with O-ring provided)
7. Ship to the BSP using the procedures described in Section 6.1

Homogenized cell lysates in QIAzol Lysis Reagent can be stored at -80 degrees C for several months.

4.5 ENDOBRONCHIAL BIOPSIES

Forceps description: Disposable forceps (Olympus FB-231D.A, required working channel 2.0mm, length 115cm, cup opening 5.0mm).

Up to 10 endobronchial biopsies (as needed to obtain 8 “acceptable” biopsies as defined below) are obtained from 2nd-through 5th-order carinae contralateral to the brushing site. Begin with 2-3 lower lobe sub-segmental carina, if patient size allows. Then proceed to

segmental carina of the lower lobes. Next proceed to right middle lobe or lingula (segmental carina then main carina). Finally, one may need to biopsy segmental or main carina of ipsilateral upper lobe to acquire 8 acceptable biopsies, however these carina are more technically difficult to biopsy due to location and cartilage content.
To take biopsies, advance forceps and close forceps over a carina (we typically choose the lateral edges of the carina, if possible, as biopsies from the center of the carina leave a larger wound). Close the forceps and hold for a seven second count. Pull back deliberately with both the forceps (assistant) and the bronchoscope (bronchoscopist's non-dominant hand). Gently remove biopsy from forceps with a tuberculin or other small bore syringe filled with saline into a cell culture dish with a small "dot" of saline. Try using saline alone to remove biopsy. Then consider using the small needle if that is unsuccessful. Examine biopsy with dissecting scope or magnifying glass for quality. Acceptable biopsies will be those that appear to have epithelium and submucosa by direct examination by magnifying glass or dissecting scope. Tips for identifying unacceptable biopsies include samples that take the form of elongated epithelial scrapings or blood clot without adequate tissue. Later, during histological examination at high power, our analyses will require at least 2 biopsies with intact epithelium and, in our experience, we need to have 6 biopsies that appear to be good on examination in the bronchoscopy suite to ensure adequate tissue at histological examination.

In the case of bleeding, 1-3mL of saline wash can be used to clear the field of view. Should bleeding prevent a clear field of view, or other safety concerns arise, the bronchoscopy should be terminated. The ordering of samples is designed to enhance collection of the most important samples in the earliest time points.

4.5.1 Biopsy Handling

Biopsy order: Biopsies will be allotted as follows. Following this order will optimize allocation should a smaller than planned number of biopsies be obtained.

a. Biopsy 1: Snap Freeze
b. Biopsy 2: Formalin
c. Biopsy 3: Formalin
d. Biopsy 4: Formalin
e. Biopsy 5: Formalin
f. Biopsy 6: Snap Freeze
g. Biopsy 7: Formalin
h. Biopsy 8: Formalin

1) **Biopsies for snap freezing and homogenization:**
    a. Carefully remove endobronchial biopsy from the forceps using a 1cc syringe attached to a 25 gauge needle and filled with 0.9% nuclease free PBS. Gently squirt the biopsy with the PBS to remove it from the forceps.
    b. Place biopsy in O-ring Aliquot tube suitable for liquid nitrogen, tightly screw-on cap and place IMMEDIATELY in portable thermos containing liquid nitrogen in the bronchoscopy suite.

c. Sample may thereafter be stored at -70 or -80 degrees C until homogenization at the GIC.
d. Homogenization will be performed in 600µL QIAzol and the homogenized sample should be transferred to a 1.5 ml aliquot with O-ring and stored at -80C. Homogenization can be performed in batches keeping samples separate and storing them at -80 degrees until shipping. Homogenized samples can be shipped centrally in QIAzol for further extraction at the GIC. Homogenization should be completed under a hood, wearing eye protection.

2) **Biopsies for Histology:**
   a. Carefully remove endobronchial biopsy from the forceps using a 1cc syringe attached to a 25 gauge needle and filled with 0.9% nuclease free PBS.
   b. Using the PBS, gently flush the biopsy to loosen it away from the biopsy forceps while using the needle to tease the biopsy away from the forceps.
   c. Once the biopsy is freed from the forceps, it is immediately placed onto a drop of nuclease free PBS in turn placed into a Petri dish. Biopsy is then carefully examined under a dissecting scope for quality. Once quality has been assured, each biopsy is carefully placed into a 20ml glass screw cap vial (a "scintillation" vial) containing 10ml ice cold 10% neutral buffered formalin (at this step all biopsies will be in the same vial for fixation) in a 20ml glass scintillation vial (Wheaton cat# 986548).
   d. Biopsies are transported to working lab and placed in 4 degrees C refrigerator and fixed for one hour at 4 degrees C in the formalin.
   e. After one hour, formalin is replaced with fresh 10% neutral buffered formalin and fixed for an additional 3 hours at 4 degrees C. To do this, pour out the formalin slowly keeping biopsies in the vial (if you pour slowly they will stick to the side of the glass vial and be left behind). Then refill the vial with 10ml of fresh, ice cold formalin.
   f. After the 4 total hours of fixation, formalin is replaced with 70% alcohol and stored at 4 degrees C until ready for paraffin processing. To do this, pour out the formalin slowly keeping biopsies in the vial. Then refill the vial with 10 ml of 70% ethanol.
   g. Biopsies will subsequently be processed in the histology or pathology core laboratory associated with each site using approved protcols (see section 4.5.2 below). We prefer that the biopsies be processed immediately by the core lab. However, if necessary, the biopsies can sit in 70% ethanol for a maximum of 2 days before processing by the core lab.
   h. The core labs will either process the biopsies into paraffin blocks or their final form will be biopsies wrapped in filter paper within a biopsy cassette (after paraffin infiltration). Eiher approach is acceptable. Whether embedded in pafaffin block(s) or as separate paraffin-infiltrated biopsies, the samples should be shipped to UCSF with a manifest indicating the number of biopsies and a cold block (to avoid melting in warm weather).

i. Please place each set of biopsies into a plastic bag and and ensure each bag is also labelled with the correct participant id and visit number.

4.5.2. Processing protocol which should be performed by your site's core histology or pathology laboratory

a. 70% ethanol for at least 15 minutes
b. 95% ethanol for at least 15 minutes
c. 95% ethanol for at least 15 minutes
d. 100% ethanol for at least 15 minutes
e. 100% ethanol for at least 15 minutes
f. 100% ethanol for at least 15 minutes
g. Xylene for at least 15 minutes
h. Xylene for at least 15 minutes
i. Xylene for at least 15 minutes
j. Melted paraffin for 1 hour
k. Melted paraffin for 1 hour

5 POST BRONCHOSCOPY MONITORING

Calculate total lidocaine amount administered. (Subtract remaining lidocaine volume from your starting volume and calculate mg lidocaine delivered.) Continue monitoring blood pressure, heart rate, respiratory rate and oxygen saturation every 15 minutes for the first hour and every 30 minutes thereafter until discharged.

If sedation not given:
- > 600 mg or 9 mg/kg whichever is less delivered - monitor ≥ 2 hours.
- < 600 mg or 9 mg/kg whichever is less delivered - monitor ≥ 1 hour.

If sedation given:
Monitor ≥ 2 hours, regardless of dose of lidocaine given.

Remove the IV line once the patient is fully alert and oriented (especially if any sedatives have been administered) and once it is certain the patient has no delayed hemoptysis. Warn the subjects that small streaks of blood may be noticed in expectorated sputum for a few hours after bronchoscopy, but that these should decrease progressively in volume.

Measure the participant's FEV_1 post-bronchoscopy. Coordinators should adhere to all local site policies regarding post-bronchoscopy spirometry. If no specific policies are in place, check the FEV_1 1.5-2 hours after completion of the bronchoscopy. If it is < 90% of the initial, pre-bronchodilator FEV_1, administer 2 -4 puffs albuterol and repeat spirometry 10 - 15 minutes later.

5.1 Suggested discharge requirements (please adhere to local guidelines)

1 Able to sip water without coughing
2 Heart rate < 100 / minute
3 FEV1 > 90% initial FEV1
4 Ambulate without difficulty
5 Volunteer is in no apparent distress

Review post-bronchoscopy instructions with the volunteer, including provision of instructions to contact the physician on call 4-6 hours after being discharged, on the following morning, and at any time the subject thinks he or she may be suffering from a complication. The subject should be informed of the possibility of a low-grade fever and a sore throat the night after bronchoscopy, but should also be instructed to call the "on-call" physician for rigors, production of purulent sputum, worsening hemoptysis, or shortness of breath not promptly relieved by albuterol inhalation. If the subject has received any sedation, he/she should be instructed not to drive for 8 hours, and should accompanied by an adult on discharge home.

Contact volunteers the night of the bronchoscopy procedure and 24 hours later. Document findings.

6 SPECIMEN SHIPPING

All specimens must be recorded in the SPIROMICS DMS on the day of collection. A tracking log should be shipped with the samples.

6.0 Storage, packaging, and shipping for shipments to the BSP

Ship frozen specimens on Mondays- Wednesdays only. Email the GIC Repository at all of the emails listed below least 48 hours in advance to insure they are ready for your shipment. In this email please include as attachments all excel shipping manifest forms (located on secure SPIROMICS website under forms) as appropriate. Shipments of bronchoscopy samples *going to the GIC* can be batched together but should not be stored locally for more than one month. Determine the number of boxes to be shipped that month and gather up the appropriate number of shipping containers needed for shipment. Each shipping container consists of a Styrofoam container surrounded by a cardboard shipping box. Approximately 16 two-inch boxes should fit per carton along with 5-8lbs of dry ice. These cartons can be recycled (the GIC-repository can send back empty cartons via ground transportation). **Note: during the packing process the aliquots and conical tube boxes should be kept on dry ice at all times.**

6.1 Packaging -80ºC Specimens

The frozen samples include: 1) Three 15mL conical tubes from the oral rinse; 2) One 15mL conical tube from bronchial wash; 3) One aliquot with pellet from bronchial wash; 4) At least 5 aliquots of supernatant from bronchial wash; 5) One 15 mL conical tube of

BAL; 6) One aliquot for alveolar macrophage isolation; 7) Forty aliquots and four 15mL conical tubes of supernatant from BAL; 8) One to three aliquots with protected brush specimens; 9) One aliquot of cytological brush specimens; 10) One aliquot of homogenized biopsies; 11) Two 15mL conicals of saline (one directly from the saline and one drawn through the scope); 12) Up to 15 aliquots of plasma from the lavender tube; 13) One sample of whole lavage for culture.; 14) Optional distal airway brushes. A separate freezer box of mircrobiome sputum samples from Bronchoscopy Visit 1 are also shipped to the BSP using the protocol below.

- Package samples on the afternoon of shipment, if done earlier maintain package at -80ºC until it is shipped.
- Include the excel spreadsheet manifest prepared 48 hours in advance (see above). Place a manifest into each of the shipping containers, and check off each item as it is packed.
- Put dry ice in a tray or other temporary container for transport of the frozen aliquot and conical tube boxes to shipping area.
- Place a rubber band around each of the frozen boxes
- Remove Styrofoam container from card board box before beginning packaging
- Place some dry ice in the Styrofoam inner container (enough to cover well the bottom of the shipping container), and begin layering boxes and dry ice into the container. Make sure all air pockets contain dry ice. Repeat for additional shipping containers as needed.
- The amount of dry ice in the shipping container should total approximately 5 pounds.
- Put one piece of strapping tap across Styrofoam box top
- Place the GIC repository address and the paper shipping manifest (with the address on top into a plastic folder or Ziploc plastic bag and tape to top of Styrofoam container.
- Put top on Styrofoam container
- Put Styrofoam box back into card board box
- Seal the cardboard box containing the Styrofoam box inside securely with strapping tape.
- Affix a Biological Substance Category B (UN3373) label and a Dry Ice label (UN 1845) to the outside of the cardboard box. Make sure that both the shipper's and sender's address is on the dry ice label. Please note these labels are not provided by the GIC.
- Affix the completed (see below for specifics) FedEx airbill or online package slip to the following address to the outside of the box. If using an airbill:
    - Record the site address and telephone number in section 1. Record the GIC's FedEx account number on the appropriate line (contact the GIC for this information)
    - Record the GIC internal billing reference in Section 2 (contact the GIC for this information)
    - Section 3 should be the GIC repository address (below):
      BioSpecimen Processing Facility

Rm. 3213 Michael Hooker Research Center
135 Dauer Drive
Campus Box 7406
The University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7406

- Section 4--**Check priority overnight**
- Section 5 (packaging) = check **other**
- Section 6 –check **Yes** shippers declaration not required and check **dry ice** and write on the estimated number of pounds
- Section 7 – Check **Third Party**
- Use your institution's FedEx shipping procedures or call 1-800-GO-FEDEX for pickup.

– Send an e-mail message containing the tracking number and date of shipment to all of the addresses below.
Heena Mehta (hmehta@med.unc.edu), Hednrik Dejong (hdejong@email.unc.edu), Patricia Basta (patricia_basta@unc.edu), and Betsy Carretta (betsy.carretta@unc.edu)

6.2 Storage, packaging, and shipping for shipments to the Immunophenotyping Core (4ºC Shipments)

The Immunophenotyping samples include: 1) Sputum samples collected at separate visit; 2) Blood collected prior to bronchoscopy; and 3) BAL samples.

Immunophenotyping specimens can be shipped on Monday - Thursday only. Email the Immunophenotyping Core (Curtis Lab) Repository at least 48 hours in advance of a scheduled bronchoscopy or sputum visit at the emails listed below to insure they are ready for your shipment. In this email please include as attachments all excel shipping manifest forms as appropriate. **Immunophenotyping samples must be shipped the day they are collected.** Use the shipping containers and supplies provided by the Immunophenotyping Core.

– Include the excel spreadsheet manifest
– Place appropriate number of frozen gel packs on bottom of Styrofoam container to line the bottom.
– Place at least a half inch of padding on top of frozen gel pack before placing the wire rack containing the specimens destined for the Immunophenotyping Core.
– Place an additional half inch of padding on top of box and place another frozen gel pack on top of box.
– Place packing material generously around gel packs and box(es).
– Place the Curtis Lab (Immunophenotyping Core) address and the paper shipping manifest (with the address on top into a plastic folder and place on top of tubes.
– Seal carton with strapping tape.

- Affix an "Exempt Human Specimens" label to the outside of the cardboard box. Please note these labels are not provided by the GIC.
- Affix the completed (see below for specifics) FedEx airbill or online shipping form to the following address to the outside of the box. If using an airbill:
  - Record the site address and telephone number in section 1. Record the **GIC's FedEx account number** (i.e., this using the same shipping account even though it goes to a different address) on the appropriate line (contact the GIC for this information)
  - Record the GIC internal billing reference in Section 2 (contact the GIC for this information)
  - Section 3 should be the GIC repository address (below):

    Curtis Lab, 11R
    Bldg 31, Rm 111
    2215 Fuller Rd
    Ann Arbor, MI 48104
    ship to phone: 734-845-5078
  - Section 4--**Check priority overnight**
  - Section 5 (packaging) = check **other**
  - Section 6 – check **No**
  - Section 7 - Check **Third Party**
- Use your institution's FedEx shipping procedures or call 1-800-GO-FEDEX for pickup.
- Send an e-mail message containing the tracking number and date of shipment to all of the addresses below:
  Betsy Carretta (betsy.carretta@unc.edu), Christine Freeman (cmfreema@umich.edu), Valerie Stolberg (vstolberg@yahoo.com), Sean Crudgington (scrudgin@umich.edu) AND Jeff Curtis (jlcurtis@umich.edu)

6.3 Storage, packaging, and shipping for shipments to UCSF (4ºC Shipments)

Bronchoscopy specimens should be shipped Monday – Wednesday only. Email the UCSF at least 48 hours in advance at the emails listed below to insure they are ready for your shipment. In this email please include as attachments all excel shipping manifest forms as appropriate. Bronchial biopsies that are paraffin embedded for histology should be stored at clinical collection site and shipped every 10 days to UCSF at the address listed below for further processing.

Samples can be batched together if more than one bronchoscopy has been performed.

- Include the excel spreadsheet manifest
- Place appropriate number of frozen gel packs on bottom of Styrofoam container to line the bottom.
- Place at least a half inch of padding on top of frozen gel pack before placing the 2" box(es) containing the specimens destined for UCSF.
- Place an additional half inch of padding on top of box and place another frozen gel pack on top of box.

- Place packing material generously around gel packs and box(es).
- Place the UCSF address and the paper shipping manifest (with the address on top into a plastic folder and place on top of tubes.
- Seal carton with strapping tape.
- Affix an "Exempt Human Specimens" label to the outside of the cardboard box. Please note these labels are not provided by the GIC.
- Affix the completed (see below for specifics) FedEx airbill or online shipping form to the following address to the outside of the box. If using an airbill:
    - Record the site address and telephone number in section 1. Record the **GIC's FedEx account number** (i.e., this using the same shipping account even though it goes to a different address) on the appropriate line (contact the GIC for this information)
    - Record the GIC internal billing reference in Section 2 (contact the GIC for this information)
    - Section 3 should be the UCSF address (below):

      Woodruff Lab: Attention Suresh Garudadri
      513 Parnassus Ave
      HSE1355A
      San Francisco, CA 94143-0130

    - Section 4--**Check priority overnight**
    - Section 5 (packaging) = check **other**
    - Section 6 – check **No**
    - Section 7 **-** Check **Third Party**
- Use your institution's FedEx shipping procedures or call 1-800-GO-FEDEX for pickup.
- Send an e-mail message containing the tracking number and date of shipment to all of the addresses below.

  Suresh Garudadri (Suresh.Garudadri@ucsf.edu)

  Betsy Carretta (betsy.carretta@unc.edu)

7 REFERENCES

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4) Thompson AB, Huerta G, Robbins RA, Sisson JH, Spurzem JR, von Essen S, Rickard KA, Romberger DJ, Rubinstein I, Ghafouri M, et al. The bronchitis index. A semiquantitative visual scale for the assessment of airways inflammation. Chest. 1993 May;103(5):1482-8.