MOP 5

SPUTUM INDUCTION AND PROCESSING

September 11, 2014
1 Summary

To safely obtain airway sputum samples with minimum contamination of oral squamous epithelial cells, and to accurately collect and store the sample prior to delivery to laboratory personnel. Sputum induction provides an opportunity to directly measure biomarker activity within the lung.

1.1 Procedure Specific Inclusion/Exclusion Criteria

Inclusion
No specific inclusion criteria.

Exclusion
- Participants with a known intolerance to albuterol or salmeterol
- Participants with a known history of poor outcomes with sputum induction.
- Participants with a post-bronchodilator FEV$_1$ of <35% during screening.

1.2 Brief testing sequence for each visit

To conduct a sputum induction subjects must not eat or drink anything (except water) for at least two hours prior to beginning the induction. Subjects must also have been able to perform three reproducible flow-volume curves during the pulmonary function testing portion of the study visit. For subjects at risk of bronchospasm, the post-bronchodilator values are acceptable.

Subjects should be coached in the proper technique for saline inhalation, coughing, and nasal/oral/pharyngeal cleansing. The coordinator then conducts the induction processes. Saline concentration levels and length of inhalation are determined by the initial spirometry. Sputum samples must be processed without delay after collection.

The sputum sample must be kept on ice during the sputum induction. If the processing unit is not in the same building and/or the specimen cannot be delivered immediately, then prior to transporting to the processing unit, the sputum cup should be placed deep into a lab bucket which is full of ice and the bucket should be covered with a lid for immediate transport.

Processing should begin within 30 minutes of collection.

1.3 Measurements

1) Mucin/water content:
   a) Total mucin concentration
   c) Muc5AC and muc5B contributions to total mucins
d) Mucus rheologic parameters

e) Mucin complex discovery proteomics-mass spec

2) Viscoelastic measurements

3. Regulators of mucus water/mucin content
   a) Nucleotides and nucleosides

3) Microbiology
   a) Bacteria
   b) Viruses

4) Cells
   a) Inflammatory

5) soluble biomarkers
   a) Cytokines
   b) PGP
   c) Discovery proteomics

6) Viral RNA

2  Equipment

2.1 Durable Supplies/Equipment

Devilbiss Ultra-Neb 99 Ultrasonic Nebulizer (or site approved nebulizer) and pulmonary function equipment. The testing must be performed in an area with a sink the subject can expectorate into.

2.1.1 Cleaning
   - Nebulizer Unit
   - BP monitor and stethoscope
   - Work table
     - Equipment should be wiped down with disinfectant, such as Sani-cloth plus germicidal disposable wipes or other institutional disinfectant

2.2 Disposable Supplies

Disposable supplies required are:
   - nebulizer cups and lids
   - disposable plastic aerosol tubing
   - mouthpiece
   - tissues
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- container with ice for sample storage
- sterile specimen cups with lids
- paper spacer for albuterol puffer
- drinking cup
- sterile syringes
- 3%, 4%, and 5% and 0.9% saline
- Albuterol

3 Personnel

3.1 Qualifications and Training

The individual performing the sputum induction should be familiar with the equipment used and all required steps in the induction procedure. The individual should also be knowledgeable about sample requirements and preparation. Persons performing sputum induction should be proficient in pulmonary function testing, and able to administer fast acting bronchodilators (albuterol) when necessary. Additionally, since many of these subjects will have Chronic Obstructive Lung Disease (COPD) and/or asthma, the technician should be able to recognize and respond to other respiratory problems which may occur with these study subjects.

Study personnel are either centrally trained, trained by individuals who have participated in central training, trained during monitoring visits, or trained via webcast. All personnel conducting SPIROMICS sputum induction and processing must be certified and maintain that certification on an annual basis.

4 Subject Preparation

4.1 Safety

Always use universal precautions when handling body fluids (wear gloves).

Subjects should never be left unattended once the procedure has started. All COPD subjects will have albuterol (2 puffs) administered prior to the procedure. Oxygen and albuterol will be available.

Communicate with the processors and study physician prior to starting.

4.1.1 FEV1 Values

Sputum induction should not be performed if the subject is unable to perform acceptable/valid spirometry trials, or if baseline spirometric indicators are below stated acceptable criteria. Participant’s whose initial FEV1 is less than 35% predicted should not be included in the sputum induction portion of SPIROMICS. If at any point during the procedure, a participant’s FEV1 decreases by 20% from baseline the procedure should be stopped.
For patients with COPD, depending on the severity of the disease, the procedure will always have the flexibility to administer “lower” doses of hypertonic saline for “shorter” durations of time, if necessary.

4.1.2 Previous problems with induction or bronchodilators
Subjects with known intolerance to albuterol or salmeterol, or who have a history of intolerance to sputum induction, should not be included in this study.

5 Procedure Steps

4.1 Nebulizer preparation:

The technician should wash his/her hands prior to preparing the nebulizer, and all procedures should be performed in a clean (not sterile) fashion. Insert the nebulizer chamber into the unit so that the notch at the bottom of the chamber is aligned with the locating rib at the front of the unit. When positioned properly, the fill label on the chamber will be facing you. Insert the RF reflector ring (metal disc with a hole in the middle and a notch on one side) into the nebulizer chamber. Fill the nebulizer chamber with tap water up to the fill line on the side of the chamber. The unit will not function if the water level is too low or too high. Place the disposable nebulizer cup and lid into the chamber. A length of disposable aerosol tubing, 2 sections or approximately 12” should be attached to the elbow for the air supply, and to the lid on the disposable cup. A second length of tubing, 5 sections, or approximately 30” should be attached to the other port on the lid of the nebulizer cup. The mouthpiece should be inserted on the other end of the aerosol tubing. Place 15ml (a total of 30 ml is made when saline is mixed for the 4% level, use entire volume) of saline at each level into the disposable cup. Turn the unit on with the switch on the lower right hand side of the cover. Adjust the output to the highest level the subject can tolerate, ideally no less than halfway.

5.2 Saline preparation:

3% saline, 0.9% saline, and 10% saline is available in 15ml disposable vials.  
- To make 4% saline, mix equal parts of 3% saline and 5% saline.
- To make 5% saline, mix 18ml of 0.9% saline and 15ml of 10% saline.

Prior to mixing the saline preparations, the technician should use good handwashing technique, and all solutions should be handled in a sterile manner (single patient use saline, mix in sterile containers, use new syringe for each subject).

In some cases, the site pharmacy will prepare the saline concentrations. In these instances it is acceptable for the pharmacy to use bulk volumes of saline to make the appropriate concentrations.

5.3 Subject preparation:
The patient should be seated in a non-rolling chair. Subjects should have last eaten 2 hrs prior to sputum induction. The coordinator conducting the sputum induction should confirm in the DMS that a complete medical history and current health status assessments were completed during the Fasting Block (i.e., first block) of the study visit. Coordinator should repeat the vital signs assessment prior to sputum induction (Heart Rate, Respiratory Rate, Temperature, and Blood Pressure) should be obtained prior to spirometry. Spirometry should not be conducted if the participant is currently or was recently ill (NOTE: Participant visit should be reschedule if they are currently or recently ill. Please see MOP 1 – Clinical Center Procedures).

Baseline spirometry should be obtained, collected with ATS/ERS standards in mind. All baseline values should be reviewed by a qualified healthcare (MD, RN, RCP) provider or knowledgeable PI prior to beginning the actual induction. Site’s may also use a peak flow meter or other device capable of assessing FEV1 in place of a spirometer if a spirometer is not available or to reduce participant fatigue.

All subjects will be treated with albuterol, and have spirometry repeated at 10 minutes post dose to obtain post-bronchodilator baseline spirometry, again following ATS/ERS standards. For participants without COPD, only those with asthma will be treated with a bronchodilator. For the actual induction, instruct the subject to relax and to inhale though his/her mouth and exhale through the nose when breathing the saline. The subject should be told to breathe semi-deep tidal breaths, but not to hyperventilate. Instruct the subject to expectorate any saliva-salt water into a separate water cup, i.e. not to swallow any build up of this fluid. This prevents any nausea or discomfort from the saline, and helps to preserve a more acceptable specimen. Describe the cleansing technique to the subject prior to starting the induction.

Subject should have fasted for 2 hours prior to the procedure except for clear liquids which are allowed (see above). No caffeine should have been consumed within the past 12 hours. However participants may have clear fruit juice (e.g., apple or cranberry juice or clear carbonated beverages without caffeine, (e.g., caffeine free seven-up or gingerale), or mild herbal/caffeine free tea. These restrictions have implications for timing of induction for coordinators. Coordinators should plan to conduct inductions after PFTs and CT scans are completed and at least two hours after lunch and/or snack have been given to the participant.

5.4 Induction procedure:

The induction procedure is dependent on the post albuterol FEV1 obtained from the subject.

5.4.1 If the FEV1 is greater than or equal to 50% predicted, then:

Put 15ml of 3% saline into the disposable nebulizer. Turn the unit on and start the timer for 7 minutes. Ensure that the subject is comfortable, and has the mouthpiece properly in his/her mouth. Adjust the output. If the subject has an urge to cough, he/she may do so,
without scraping, and expectorate the sample into the sterile cup labeled with the subject number.

At the end of the 2 minutes, stop the timer and turn off the nebulizer, and have the subject come off the mouthpiece. Be careful not to spill or drain the saliva into the nebulizer - the saliva can build up in the tubing.

Perform spirometry. It is acceptable to only obtain one effort at this point if it is technically acceptable, and if it falls into the required range. This is to avoid subject fatigue. If there is any question about the quality of the effort, then allow the subject another minute or so of rest and repeat.

If the FEV1 has not fallen 10% or more, restart the timer and the nebulizer, and continue until the 7 minutes have elapsed. Bring the subject to the sink, and have him/her to rinse mouth and gargle thoroughly and then spit into the sink. The subject should be told to clear his/her throat, i.e. scrape the back of the throat and roof of mouth (demonstrate), and again expectorate this into the sink. The subject then blows his/her nose and discards.

Finally, have the subject give a good cough effort from the chest and without scraping the throat, expectorate the sputum into the cup. DO NOT HOCK OR SCRAPE when producing the sample. Passively bring it past the throat into the cup. Once the subject can no longer bring up sputum, repeat spirometry. If the FEV1 falls less than 10 %, dispose of the remaining saline, and put 15ml (or total amount of saline when mixed) of 4% saline into the cup. Repeat the procedure for 2 minutes then stop the clock and repeat the spirometry. If the FEV1 is stable continue with the 4% saline till the 7 minute mark. Repeat the cleansing and cough steps followed by spirometry. Again, if the FEV1 falls less than 10% then continue with 15ml of 5% saline for 2 min. then stop the clock.

Repeat the spirometry. If the spirometry drops less than 20% continue to completion and repeat cleansing, cough and final spirometry.

**5.4.2 If the FEV1 is less than 50% predicted, then:**

Put 15ml of 0.9% saline into the disposable nebulizer. Turn the unit on and start the timer for 7 minutes. Ensure that the subject is comfortable, and has the mouthpiece properly in his/her mouth. Adjust the output. If the subject has an urge to cough, he/she may do so, without scraping, and expectorate the sample into the sterile cup labeled with the subject number.

At the end of 1 minute, stop the timer and turn off the nebulizer, and have the subject come off the mouthpiece. Be careful not to spill or drain into the nebulizer the saliva which typically builds up in the tubing.

Perform spirometry. If the effort is technically acceptable, obtain one effort at this point if it falls into the required range. This is to not fatigue the subject. If there is any
question about the quality of the effort, then allow the subject another minute or so of rest and repeat. If the fall in FEV1 is less than 20%, restart the timer and the nebulizer, and continue until 2 minutes have elapsed.

Repeat spirometry. Again, if the fall in FEV1 is less than 20% continue the induction until 5 minutes have elapsed. Repeat spirometry. If the fall in FEV1 is still less than 20% continue until the full 7 minutes have passed. Bring the subject to the sink, and have him/her to rinse mouth and gargle thoroughly and then spit into the sink. The subject should be told to clear his/her throat, i.e. scrape the back of the throat and roof of mouth (demonstrate), and again expectorate this into the sink. The subject then blows his/her nose and discards. Finally, have the subject give a good cough effort from the chest and without scraping the throat, expectorate the sputum into the cup. DO NOT HOCK OR SCRAPE when producing the sample. Passively bring it past the throat into the cup.

Repeat spirometry. If the fall in FEV1 is less than 10%, dispose of the remaining saline, and put 15ml (or total amount of saline when mixed) of 3% saline into the cup. If the fall in FEV1 is between 10%-19% add a new 15ml of 0.9% saline. Repeat the same procedure as above for another 7 minutes, i.e., performing FEV1 checks at 1, 2, 5 and 7 minutes as before. If the fall in FEV1 is less than 20% at 1, 2 and 5 minutes, continue the procedure until the 7 minute mark then perform spirometry. If the fall in FEV1 is less than 20% following the 7 min. mark, put in 15 ml of the same saline concentration used for the second 7 min. inhalation to ready the nebulizer for the final 7 minute inhalation period. Now perform the cleansing and cough procedure. Next, advance to the final 7 min. inhalation period by performing spirometry at 1, 2, 5 and 7 min as you've done before. Following spirometry at the 7 min. mark, perform the final cleansing and cough procedure.

5.5 Important Notes

If the spirometry drops between 10%-19% of post-bronchodilator baseline you may continue the test, lower the saline concentration, but never increase the concentration of saline.

The test is always terminated immediately when the FEV1 drops by 20% or more, or if the subject becomes distressed and requests that the test be terminated. Once a test is terminated it may not be re-started under any circumstances. Always be prepared to administer a dose of albuterol if necessary. If a second dose of albuterol is given, perform spirometry after 10 minutes. The subject should not be discharged unless the FEV1 is within 10% of baseline. Prior to discharge assess the subject (lung sounds) and assess vital signs - ensure vital signs are within normal range. If they are not, contact the physician in charge immediately.

The sputum sample should be kept on ice throughout the induction procedure. A second specimen cup labeled "waste" should be used during the procedure to capture saliva and spit that may build up in the subjects' mouth. This waste material should not be
expectorated into the "sample" specimen cup. The subject may take the cup out of the ice bucket to expectorate into, but it should go back on ice immediately afterwards.

Deliver the sample to the processing lab without delay.

### 5.6 Recordkeeping:

Spirometry data should be recorded on the worksheet for each induction. A qualified healthcare provider should review and sign all worksheets.

### 5.7. Cleaning and infection control:

Use a new disposable nebulizer cup for each subject each day. Wipe the exterior of the unit with alcohol or another surface disinfecting solution. Change the disposable aerosol tubing between subjects. The nebulizer chamber should be removed from the unit and drained each day.

### 6 Risks to Human Subjects/Safety Issues

6.1 Possible Adverse Events

*Risks with hypertonic saline:* Inhalation of hypertonic saline for sputum induction may result in wheezing, coughing or chest tightness particularly in susceptible individuals such as those with asthma. Asthmatic subjects in our study will be pre-medicated with albuterol in order to minimize this risk. Spirometry will be evaluated for all subjects before induction as well as at prescribed intervals during each of the 3 levels of hypertonic saline. Subjects may also experience transient throat irritation during the hypertonic saline inhalation but this generally resolves post induction when the subject is provided with a snack and juice or water.

*Risks with albuterol:* Risks associated with albuterol use in this study are minimized by excluding subjects with serious concomitant illnesses or risk factors for chronic illness including but not limited to individuals with cardiovascular disease, diabetes, hypertension, active arrhythmias or thyroid disease, glaucoma, and individuals older than 50 years. Study subjects may most commonly notice short lived increase in heart rate and mild tremor as a result of albuterol use.

6.2 Procedure Termination

The procedure is terminated 1) if the FEV₁ falls by > 20% at any time point, or 2) if the subject requests that the procedure be stopped, or 3) after three, seven-minute inhalation periods have been completed.
6.2 Central Quality Assurance (GIC and/or Reading Center)
   6.2.1 Site Visits
   6.2.2 Training/retraining
   6.2.3 Equipment validation
   6.2.4 Procedure verification

9. Sputum Processing, storage, and shipping

9.1 Sample Processing

Sputum samples will be processed using one of two methods. Which method is used is determined by the **sample weight**. If the initial sample weight (recorded in SPW01) is greater than or equal to 2.5g, Method 1 is used. If the specimen is less than 2.5 g, Method 2 is used.

   Method 1 (Samples $\geq 2.5$g, Original Method) – Process sample by first removing three aliquots per Sections 9.1.2 (mucin aliquot), 9.1.3 (microbiology aliquot), and 9.1.4 (viscoelastic aliquot) before adding EDTA in Section 9.1.5.

   Method 2 (Samples <2.5g, Direct-to-EDTA Method) – After Section 9.1.1 (weighing the sample) proceed to adding EDTA as in Section 9.1.5 (i.e., skip Sections 9.1.2 – 9.1.4)

Indicate which method you are using in Question 6a on the Sputum Processing Worksheet (SPW).

9.1.1 Weighing of Sputum Sample

1) Pour entire sample into Petri dish and record color (clear, yellow green) and characteristics (visible plugs, aggregates, foamy, viscous) – do not spend more a minute or two on this
2) Weigh an empty 50mL tube with lid and record weight on it
3) Zero the balance
4) Pour sample into the tube and measure the total weight in grams
5) Put sample on ice. Steps 9.1.2 through 9.1.5 draw off this sample

9.1.2 Processing Whole Sample using the Mucin Method

1) Weigh an empty microcentrifuge tube
2) Zero the balance
3) Measure 0.500g of whole sputum sample from 1) above (if there is a small amount of sputum, you can go as little as 0.100g.
4) Record weight of sputum
5) Add 1.0 mL of 6M guanidine reduction buffer (if less than .500g sputum, add 0.5mL of Guanidine Reduction Buffer) - mix (mild vortex/tip back and forth manually) until sample is reasonably homogenized

6) Store sample at two to eight °C in box labeled SPIROMICS Mucin

9.1.3 Processing Microbiology Sample

1) Weigh an empty microcentrifuge tube
2) Zero the balance
3) From remaining sample in 9.1.1 above, measure 0.300g of whole sputum sample
4) Record weight of sputum and store at -80 deg C and ship on dry ice

9.1.4 Processing the Viscoelastic Sample

1) From remaining sample in 9.1.1 above, obtain a minimum of 25 microliters of sample. Suggest using a 100 ul pipette and draw up whatever can be achieved (exact volume is not important but using a 100ul pipette will ensure the 25 ul minimum has been achieved–deliver sample into an eppendorf tube and immediately stored at -80 deg C.

9.1.5 Processing whole sample using 1 mM EDTA for cytokines and nucleotides

1) From remaining sample in 9.1.1 above, weigh entire remaining sample and record weight in grams
2) Add 0.1% Sputolysin (DTT) in mLs equal to 4X selected sputum sample weight in grams. (For example, 2g of sample would need 8mL of 0.1% Sputolysin added) (NOTE: DTT comes as a 1% solution in 10mL vials. Make the 0.1% solution by diluting 1:10 with 1 mM EDTA).
3) Vortex sample 15 seconds and pipet up and down with P1000 pipettor to break up any clumps (this is especially necessary for very viscous/thick samples).
4) Place sample on tumbler for 15 minutes (NOTE: Timing is important).
5) Dilute sample with 1mM EDTA. Use the same volume that was added in step 1. (For example, if 8mL of Sputolysin was added, you would add 8mL of 1mM EDTA
6) Continue to tumble for an additional 5 minutes
7) Filter sample into a new 50mL tube through 53µm nylon mesh filter (from Small Parts, Inc.) lining a funnel (pre-wet the filter with buffer for better adhesion to the funnel).
8) Spin down cells at 500Xg for 10 minutes.
9) Save 4 aliquots of supernatant in 1.5mL tubes, place in participant specimen box 2 of 2 as described in MOP 4 – Biospecimen Collection and Processing and freeze in -80
10) Save 4 aliquots of supernatant in 1.5mL place in participant specimen box 2 of 2 as described in MOP 4 – Biospecimen Collection and Processing and freeze in -80
11) Add 1 or 2mL of HBSS to resuspend cells, making sure to record volume for cell count.
9.1.6 Cell Counts

1) From last step in 9.1.5 above, combine 10µL of resuspended sample with 10µL of trypan blue stain
2) Place 10µL of mixture on the hemacytometer.
3) Count live (clear) cells and dead (blue) cells in each of the 4 corner grids. (NOTE: Exclude RBC’s and note the number of squamous epithelial cells as this can be used to judge the quality of the sample – a high number of squamous epithelial cells indicates a poor quality sample and will require returning to the filtration step in 5) above and commencing from that point forward).
4) Total Cell Count (TCC) = [(sum of 4 grids/4) X 2 X 10^4] X Volume of Sample.
5) Determine number of cells/mg (=TCC/weight of selected sample)
6) Viability=(live cells/total cells) X 100%

9.1.7 Cytospins

1) From 9.1.6 above, spin down cells and remove or add HBSS to give final concentration of 1X10^6 cells/mL; mix to resuspend.
2) Make 4 slides (if possible, you must make at least one slide) using 60µL of cell suspension (single cytofunnel recommended)
3) Spin for 6 minutes at 450rpm
4) Fix and stain 2 slides with Hema 3 stain set (ten dips in each) and rinse in distilled water
5) Allow to air dry and then mount with Cytoseal and coverslip
6) Store slides in SPIROMICS box at room temperature

9.1.8 Preparing cells for extraction of total RNA

1) From remaining cell pellet in 9.1.7 above, spin down cells at 500Xg for 5 minutes and discard HBSS
2) Add 1mL of Trizol reagent to cell pellet
3) Add 10uL of GGD
4) Pipet up and down to break up cells
5) Store sample at -80°C for later extraction of total RNA

9.2 Sample Storage and Shipping

Please see Biospecimen Collection and Processing MOP (MOP 4) for details on specimen storage and shipping.

10. REFERENCES:

1. Pin, I; Use of Induced Sputum cell counts to Investigate Airway Inflammation in Asthma. Thorax 1992; 47 : 25-29
3. DeVilbiss ULTRA-NEB®99 Ultrasonic Nebulizer Instruction Guide
Appendix 1: EDTA Formulation

The molecular weight of EDTA is 372.

1. Weight 0.186g of solid EDTA and add it to 500mL of 1X PBS buffer to make 1mM EDTA solution
2. Mix solution until homogenized.