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1. Study overview

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1.1 Design and outcome measures

Title

Broccoli Sprout Extracts Trial (BEST)

Objective

To test the hypotheses that daily ingestion of 25 or 150 micromoles of sulforaphane (broccoli sprout extract) for 4 weeks will have the following dose-dependent effects:

1. Increase Nrf2 activation in alveolar macrophages and bronchial epithelial cells
2. Increase phase II anti-oxidant enzymes in alveolar macrophages and bronchial epithelial cells
3. Reduce markers of oxidative stress in expired breath condensate

Type of study

- Phase II clinical trial
- Randomized, double-masked, placebo-controlled, 3 arm parallel group clinical trial
- Multicenter, 3 centers
- Fixed sample size, 90

Study drugs

- Low dose group (25 micromoles of Sulforaphane)
  3 capsules per day by mouth
- High dose group (150 micromoles of Sulforaphane)
  3 capsules per day by mouth
- Placebo group (microcellulose)
  3 capsules per day by mouth

Primary outcome

- Nrf2 levels and downstream anti-oxidants
  Change in Nrf2 expression in alveolar macrophages and bronchial epithelial cells
  Change in phase II anti-oxidant gene expression (NQ01, HO-1, GPX2, GCLM, GCLC, GSTA1).

Secondary outcomes

- Measures of oxidative stress
  Oxidant stress indicators (isoprostane) measured in plasma and expired breath
- Measures of airway inflammation
  Measured as BAL cell counts and cytokine profiles
- Pulmonary function tests
  Spirometry, lung volumes, DLCO to determine whether there are any short-term functional effects of the treatment.
- Patient reported outcomes
  MRC dyspnea scale and SGRQ which includes questions on cough and sputum production.
- Adverse events
  Both spontaneous reports as well as targeted symptoms related to respiratory and GI systems. Adverse events scored based on the NCI Common Toxicity Criteria rating scale.
- Safety measures
  Baseline (V1) and follow-up (V4) CBC and biochemical profile including renal function, thyroid function, and liver function tests.

1.2 Eligibility criteria

The general goal of patient selection is to enroll patients who meet GOLD criteria for COPD and who are likely to tolerate repeated bronchoscopies.

Inclusion criteria

- Gender and age
  Males and females, age 40 years or older
- Physician diagnosed COPD
- Lung function criteria
  Post bronchodilator FEV1/FVC ratio < 0.70
  FEV1 40-80 % predicted
- Smoking status
  10 or more pack-years smoking history
  Both active and former smokers
- Other
  Willingness to ingest no more than 1 serving of cruciferous vegetables (e.g. broccoli, cabbage, collard / mustard greens, kale, brussel sprouts, radishes) per week during run-in and treatment periods
  Ability and willingness to provide informed consent

Exclusion criteria

- Pulmonary disease
  COPD exacerbation within preceding 6 weeks requiring treatment
- Other illnesses
  Significant respiratory (other than COPD), cardiovascular, neuropsychiatric, renal, gastrointestinal, or genitourinary disease that would interfere with participation in the study or interpretation of the results.
- Pregnancy
  Cannot participate if pregnant or lactating
  Females of childbearing potential that are unwilling to practice an adequate birth control method (abstinence, combination barrier and spermicide, or hormonal)
- Cancer
  Cancer (other than skin or localized prostate) within preceding 5 years
- Coronary Disease
  Acute MI or Acute Coronary Syndrome within 6 prior months

- Others
  - ☐ Allergy to local anesthesia
  - ☐ Resting hypoxemia (O_2 saturation <90%)
  - ☐ Glomerular filtration rate (GFR) < 30mL/min
  - ☐ Liver enzymes four times upper normal

1.3 Study visit time windows and data collection schedule

Visit	V1	V2	V3	P1	P2	V4	V5	P3
Time window (days)	-28 to -7	-14 to 0	0	1	7-14	21-30	22-31	24-38
Target (days)	-21	-14	0	1	14	25	26	27
Consent, eligibility evaluation	•							
Screening Questionnaire	•							
Spirometry	•	•				•		
Pulse Oximetry	•	•						
Lung volume/DCO		•				•		
CBC, Chemistry panel, TSH	•					•		
Urine analysis	•					•		
Physical Exam		•				•		
Pregnancy Test	•					•		
Randomization			•					
Health Status / AE		•				•		
SGRQ/MRC		•				•		
ATS-DLD		•						
EBC		•				•		
PBMC/Plasma/Serum		•				•		
Nasal Brush			•				•	
BAL/Bronchial Brush			•				•	
Adherence Counseling				•	•			
Exit interview								•

KEY: AE = adverse event questionnaire, ATS-DLD = ATS-DLD respiratory questionnaire, BAL = Bronchoalveolar lavage, CBC = complete blood count, DCO = Diffusion capacity for Carbon monoxide, EBC = Expired Breath Condensate, MRC = Medical Research Council Dyspnea Scale, PBMC = Peripheral Blood Monocyte Collection, SGRQ = St Geroge's Respiratory Questionnaire, TSH = Thyroid Stimulating Hormone, UA = Urinalysis

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2.1 Visit 1 – Screening

Overview

- V1 consists of the initial screening visit, assessment of lung function, collection of blood for Complete Blood Count (CBC), biochemistry panel, and thyroid stimulating hormone (TSH), collection of urine for urinalysis, and a pregnancy test for women of child-bearing potential.

Time frame

- Target – 21 days prior to Randomization (V3)
- Window – 1 - 4 week(s) prior to Randomization (V3)
- Time period for completing V1 tasks – 1 week
- Duration of V1 – approximately 2 hours

Equipment/materials

- Participant supplies
  - Insulated bag with gel pack
  - BEST Wallet card
  - Cruciferous flash card
  - Schedule of Visits (SOV)
- Blood/urine collecting materials as appropriate for local hospital
- Pregnancy testing kit (if applicable)
- Spirometer
- V1 forms

Tasks

- Explain study to participant
- Review consent with participant
- Sign and date consent
- Ask participant if a spirometry was done within the past 6 months. (Refer to section 4.1.1 for lung function criteria tests conducted in BEST)
- Ask participant if initial spirometry, blood and urinalysis may be sent to their primary COPD doctor. If yes, have participant complete a release statement as applicable (see sample release statement in 2.1.1 section of MOP)
- Assign participant an ID using the next sequentially numbered label on Clinic Label Sheet (see example in 2.1.2 section of MOP)
  - Register participant number in BEST data system
  - Place label in box on item 2 of Screening Form (SC)
- Complete Screening form (SC)
  - Some eligibility requirements may not be met at V1 (eg. COPD exacerbation within 6 weeks), however patient can still continue to V2 if it is anticipated they will be able to meet all eligibility criteria at time of randomization at V3
- Complete Baseline History form (BH)

- Complete Participant Information Sheet (PI)
- Collect PFT and oximetry data and record on Screening Form (SC)
- Conduct pregnancy test, if applicable
- Collect blood for CBC, Chemistry panel and TSH; send to hospital lab for analysis
  - Collect no more than 10mL of blood
  - Chemistry panel includes electrolytes (NA, K, Cl, HCO3), Glucose, BUN, Creatinine, liver enzymes (ALT, AST, Alk Phos), Bilirubin, Total protein, Albumin, Calcium
- Collect urine for urinalysis; send to hospital lab for analysis
- Distribute
  - BEST Schedule of Visits (SOV) with dates and times for all subsequent visits recorded, if possible
  - List of cruciferous vegetables flashcard
  - BEST Wallet card
- Data enter Screening form (SC) into the data system immediately after visit. This form must be data entered before a participant can be randomized at Visit 3.
- Data enter remaining forms within 10 working days
- Complete Lab Data form (LD) as soon as results of CBC, Chemistry panel, TSH and urinalysis are received from hospital lab. Data enter LD form within 10 working days.

Forms (abbreviation)

- Screening form (SC)
- Baseline History (BH)
- Lab Results (LD)
- List of cruciferous vegetables flashcard
- Schedule of Visits (SOV)

2.1.1 Prototype letter: Participant release to send spirometry, blood and urinalysis results to COPD provider

Title:	Broccoli Sprout Extracts Trial (BEST)
Protocol No.:	IRB Name Protocol # xxxx
Sponsor:	National Institutes of Health (NIH) / National Heart Lung Blood Institute (NHLBI) Bethesda, Maryland United States
Investigator:	Robert A. Wise Johns Hopkins University 5501 Hopkins Bayview Circle Baltimore, MD 21224 (410) 550-0545 (410) 905-5688 (24-hour pager)
Site(s):	Johns Hopkins University Bloomberg School of Public Health BEST Coordinating Center Johns Hopkins Center for Clinical Trials 615 North Wolfe Street, Room W5010 Baltimore, MD 21205
STUDY-RELATED PHONE NUMBER(S):	Research study questions Janet Holbrook, MPH, Ph.D. (410) 955-0930 Research-related injury Robert Wise, M.D. (410) 550-0545 (410) 905-5688 (24-hour pager)

Permission to Notify Physician:

I give permission for study personnel to fax/send my spirometry test, blood and urinalysis results to my COPD care physician.

_____ _____

Printed Name of Subject Date

_____	_____
Signature of Person Conducting Informed Consent Discussion	Date
_____	_____
Signature of Investigator (if different from above)	Date

2.1.2 Example of Clinic Label Sheet

Clinic Label Sheet	**BEST** Clinical Center Z: **TEST**	To assign participant ID: Attach next sequential label to #2 on Screening Form (SC)
BZ100	BZ101	BZ102
BZ103	BZ104	BZ105
BZ106	BZ107	BZ108
BZ109	BZ110	BZ111
BZ112	BZ113	BZ114
BZ115	BZ116	BZ117
BZ118	BZ119	BZ120

2.2 Visit 2 - Eligibility

Overview

- During V2, eligible participants will perform PFT testing, lung volumes, diffusing capacity and will complete general health questionnaires and respiratory specific questionnaires. A brief physical examination will be performed. Baseline biological specimens including blood for PBMCs will be collected.

Time frame

- Target – 14 days prior to Randomization (V3)
- Window – 14 days to 1 day before Randomization (V3)
- Time period for completing V2 tasks – 1 week

Equipment/materials

- EBC collecting and processing materials
- Blood collecting materials, as appropriate, for local hospital
- Standard PFT equipment
- Labels
    - EBC cryovial labels
    - PBMC vacutainer labels
    - Serum vacutainer labels
    - Plasma vacutainer labels
    - Nasal brushing falcon tube labels
    - Bronchial brushing falcon tube labels
- V2 forms

Tasks

- Have participant complete self-administered questionnaires
    - St. George's Respiratory Questionnaire (SG)
    - ATS-DLD Questionnaire (AT)
- Collect interval medical history data and complete Clinic Visit form (CV)
- Complete Eligibility form (EG)
- Complete Lung function tests (Spirometry, lung volume and Diffusion capacity (DLCO)) and pulse oximetry. Record on Pulmonary Function Testing form (PF). (Refer to section 4.1 for lung function test)
- Conduct brief physical exam (conducted by study physician or designee) and complete Physical Exam form (PE)
- Collect expired breath condensate (EBC). Process specimen or transfer to local BEST lab. (Refer to section 4.3 for EBC collection)
- Collect blood for PBMC, plasma and serum specimens. Process specimens or transfer to local BEST lab. Complete Blood Processing form (BP). (Refer to section 5 for specimen processing and shipping)

- Distribute Instructions for bronchoscopy. Review schedule and instructions for bronchoscopy
- Data enter forms within 10 working days

Forms and questionnaires (abbreviation)

- St. George's Respiratory Questionnaire (SG)
- ATS-DLD Questionnaire (AT)
- Clinic Visit form (CV)
- Eligibility form (EG)
- Physical Exam (PE)
- Pulmonary Function form (PF)
- Blood Processing (BP)

2.3 Visit 3 – Bronchoscopy and Randomization

Overview

- Participant will have baseline bronchoscopy for collections of bronchoalveolar lavage cells (BAL) and nasal and bronchial epithelial brushings. Randomization eligibility will be established and participant will be assigned to treatment. After recovery from bronchoscopy sedation, the participant will be given the assigned treatment

Time frame

- Target – 1 day after Visit 2
- Window – 1 to 14 day(s) after Visit 2
- Duration of Visit 3 – 4 hours

Equipment/materials

- Standard bronchoscopy equipment
- Brushes for nasal and lung brushings
- Collection containers for BAL fluid and brushing specimens
- Labels
  - Nasal brushing falcon tube labels
  - Bronchial brushing falcon tube labels
  - BAL fluid collection container labels
- V3 forms

Tasks

- Complete Pre-bronchoscopr review worksheet
- Perform bronchoscopy and collect BAL fluid, nasal and bronchial brushings as outlined in section 4.2
- Complete BAL/Bronchial Brushings/Nasal Brushings form (BL)
- Transfer all specimens to BEST processing lab
- Complete BAL Processing form (BB) – completed by BEST processing lab
- After successful bronchoscopy randomize participant
- A successful bronchoscopy is defined as
  - if the patient underwent sedation for the procedure and at least some of the specimens such as nasal or bronchial brushings were obtained, and the clinic physician does not conclude that it would be unsafe for the patient to have the second procedure.
- If participant meets all the eligibility criteria, complete the Randomization form (RZ)
  - Data enter RZ form and receive randomization assignment for participant
- Distribute treatment to participant
  - After participant has recovered from bronchoscopy sedation, give study treatment blister pack and freezer bag
  - Record distribution on Blisterpack Dispensing and Capsule Counting form (DD)
  - Record use of study treatment kit on Blisterpack Accountability Log (DK)

- Data enter all forms within 10 working days

Forms (abbreviation)

- BAL/Bronchial Brushings/Nasal Brushings form (BL)
- BAL Processing form (BB)
- Randomization form (RZ)
- Blisterpack Dispensing and Capsule Counting form (DD)
- Blisterpack Accountability Log (DK)

2.4 Phone Visit 1

Overview

- Participant is contacted by phone, the day following the baseline bronchoscopy to determine whether they have had any adverse effects related to the broncoscopy, to review instructions on the use of the study treatment and to confirm future appointment dates and times

Time frame

- Target – 1 day after Visit 3
- Window – 1 week
- Duration of phone call – 15-30 minutes

Tasks

- Conduct telephone interview using Phone Contact 1 form (P1)
  - If participant cannot be reached on the day after V3, then form should be completed as soon as participant can be reached upto 1 week after V3
  - *Note: Do not complete a second PC form for any additional unscheduled calls*
- Screen for adverse events from bronchoscopy
- Remind participant to take study treatment. Discuss any potential problems
- Confirm date of next phone call (P2)

Form (abbreviation)

- Phone Contact 1 (P1)

2.5 Phone Visit 2

Overview

- Participant is contacted by phone 1 – 2 weeks after baseline bronchoscopy (Visit 3) to determine whether they have had any adverse experiences related to study treatment and to re-instruct them on use of study treatment

Time frame

- Target – 14 days after Visit 3
- Window – 7-14 days after Visit 3
- Duration of phone call – 15-30 minutes

Tasks

- Conduct telephone interview using Phone Contact 2 (P2)
- Screen for adverse events from study treatment
- Discuss compliance with study treatment.
- Confirm date of next clinic visit (Visit 4)

Notes

- If Phone Contact 1 was not performed, complete Phone Contact 1 (P1) form and P2 form during Phone Contact 2

Form (abbreviation)

- Phone Contact 2 (P2)

2.6 Visit 4

Overview

- The participant will perform the baseline spirometry including lung volumes and diffusion capacity. Specimens will be collected for CBC, pregnancy test, urinalysis, TSH and biochemistry panel. Participant will be interviewed about follow-up health status and adverse events. A brief physical exam will be conducted. Follow-up collections of blood for biological measure will be obtained.

Time frame

- Target – 25 days after randomization (Visit 3)
- Window – 21 to 30 days after randomization
- Time period for completing V4 tasks – 7 days
- Duration of V4 – 2-4 hours

Equipment/materials

- EBC collecting and processing materials
- Blood/urine collecting materials, as appropriate, for local hospital
- Standard PFT equipment
- Pregnancy testing kit (if applicable)
- EBC cryovial labels
- PBMC vacutainer labels
- Serum vacutainer labels
- Plasma vacutainer labels
- Nasal brushing falcon tube labels
- Bronchial brushing falcon tube labels
- V4 forms

Tasks

- Have the participant bring the study capsules.
- Have participant complete self-administered St. George's Respiratory Questionnaire (SG)
- Collect information about interval medical history, adverse events and treatment adherence; complete Clinic Visit form (CV)
- Complete spirometry, lung volume and DLCO. Record on Pulmonary Function form (PF)
- Complete pregnancy test, if applicable
- Collect blood for CBC, Chemistry panel and TSH; send to hospital lab for analysis; Complete Lab Results form (LD) as soon as results of CBC, Chemistry panel, TSH and urinalysis are received from hospital lab. Data enter LD form within 10 working days
- Collect urine for urinalysis; send to hospital lab for analysis

- Collect blood for PBMC, plasma and serum specimens. Process specimens (see sections 5.2 – 5.4 of MOP for instructions) or transfer to local BEST lab. Complete Blood Processing form (BP)
- Collect expired breath condensate (EBC). Process specimen or transfer to local BEST lab
- Complete physical exam (conducted by study physician or designee) and complete Physical Exam form (PE)
- Review schedule and instructions for bronchoscopy
- Data enter forms within 10 working days
- Complete Lab Results form (LD) as soon as results of CBC, Chemistry panel, TSH and urinalysis are received from hospital lab.
- Data enter LD form within 10 working days

Forms (abbreviation)

- St. George's Respiratory Questionnaire (SG)
- Clinic Visit form (CV)
- Pulmonary Function form (PF)
- Blood Processing form (BP)
- Physical exam form (PE)
- Lab Data form (LD)

2.7 Visit 5 – Bronchoscopy

Overview

- Participant will have a follow-up bronchoscopy for collection of bronchoalveolar lavage, nasal brushings, and endobronchial brushings. Participant will return unused capsules for pill counts

Time frame

- Target – 25 days after Visit 3 (bronchoscopy)
- Window – 22 to 31 days after Visit 3
- Time period for completing Visit 5 tasks – 1 week
- Duration of visit 5 – 4 hours

Equipment/materials

- Standard bronchoscopy equipment
- Brushes for nasal and lung brushings
- Collection containers for BAL fluid and brushing specimens
- Nasal brushing falcon tube labels
- Bronchial brushing falcon tube labels
- V5 forms

Tasks

- Collect unused study capsules
  - Record number of remaining pills on BlisterpackDispensing and Capsule Counting form (DD)
- Perform bronchoscopy as outlined in section 4.2
- Collect nasal brushings specimen
- Collect BAL specimen
- Collect BAL brushings specimen
- Complete BAL/Bronchial Brushings/Nasal Brushings form (BL)
- Transfer all specimens to BEST processing lab
- Complete BAL Processing form (BB) – completed by BEST processing lab
- Data enter all forms within 10 working days

Forms (abbreviation)

- BlisterpackDispensing and Capsule Counting form (DD)
- BAL/Bronchial Brushings/Nasal Brushings form (BL)
- BAL Processing form (BB)

2.8 Phone Visit 3

Overview

- Participant is contacted by phone, the day following the final bronchoscopy to determine whether they have had any adverse experiences related to broncoscopy and to conduct an exit interview

Time frame

- Target – 1 day after Visit 5 (bronchoscopy)
- Window – 24 to 38 days after Visit 3
- Duration of phone call – 15-30 minutes

Tasks

- Conduct telephone interview using Phone Contact 3 form (P3)
- Screen for adverse events from bronchoscopy
- Conduct exit interview
- Thank patient for their participation in the BEST trial
- Send sealed Unmasking Envelope and Exit letter (see example in section 6) to participant

Form (abbreviation)

- Phone Contact 3 (P3)

2.9 Missed procedures/missed visits

Time frame (for randomized participants)

- After time window has closed for a specified study visit or phone contact and the following were missed:
  - All requirements for a visit or phone contact (missed visit)
  - One or more of the procedures or forms required for a visit (missed data at a visit)

Tasks

- Attempt to schedule next visit or phone contact at a more convenient time, preferably early in the time window
- If you are unable to contact participant
  - Attempt to contact at all telephone numbers listed (home, work, cell) on Participant Information (PI) form
  - Make calls at different times of the day
  - Attempt to e-mail participant
  - Send a letter to participant
- Record specific procedures missed or reason visit or phone contact was missed on Missed Data (MD) form

Participants who have missed one or more visits or phone contacts

- Once randomized, a participant is never considered "off study" until after the scheduled follow-up period ends; i.e., after P3 window closes
- A Missed Data form (MD) is required at the close of each time window of visits missed
- Continue attempts to contact participant unless told not to contact

Participants who refuse a second bronchoscopy

- Ask if the participant is willing to continue taking study medication and willing to have a blood collection and nasal brushing
- If yes, collect nasal brushing at Visit 4 or a special appointment after Visit 4

Study medication

- If a participant extends visits beyond 31 days and uses up all study medication, issue a second blister pack
- With an extra blister pack a participant can continue up to 2 months before the second bronchoscopy
- A bronchoscopy may be performed up to 7 days after study medication is finished

Form (abbreviation)

- Missed Data form (MD)
- Participant Information (PI)

2.10 Rescreening and recycling

Rescreening

Purpose

- To re-evaluate patients who did not meet eligibility criteria during screening at V1

Tasks

- Maintain folder on patients who were not able to meet entry criteria at V1, but who may be eligible in the future
- After a 4 week waiting period you may try to re-screen a participant
- Use the participant ID number and name code as originally assigned
- Contact DCC to determine if V1 procedures need to be repeated
- If participant is eligible, enter new forms into the database. NOTE: If any form from failed screen was data entered. (DO NOT delete this form from the database)
- There is not a limit on the number of times a patient can be re-screened, but clinics should use their discretion as to what is reasonable

Recycling

Purpose

- Attempt to randomize a participant into the trial who previously failed to meet eligibility criteria at Randomization (V3) visit

Tasks

- If RZ failure due to an COPD exacerbation between V2 and V3, contact DCC with details of event. Possibility of recycling to be determined on a case by case basis

2.11 Scheduling visits

Visit 1

- Spirometry
  - Pre and post spirometry should be performed at Visit 1. However, if the patient took a short-acting bronchodilator within 4 hours or a long acting bronchodilator within 12 hours of testing, do not perform post BD testing. The information will be considered "missing", but the visit may continue
  - Clinics cannot ask a patient to "withhold" medication before a patient has signed a consent form. However, if a patient has an early morning clinic appointment, the patient can wait to take their morning medication at the clinic after spirometry testing.

Visit 2

- Combining Visit 1 and Visit 2
  - Visit 1 and Visit 2 may be performed in one day. However the following points must be followed:
    - Participant should be on a low cruciferous vegetable diet for at least 1 week before randomization at Visit 3
    - Visit 2 PFT testing is required. Both pre and post FEV1 should be performed
    - Some items on the Clinic Visit (CV) form refer to the time since the last clinic visit. If Visit 1 and Visit 2 are combined, consider the last one week when responding to these items
    - Study physician should review lab results of CBC, Chemistry panel, TSH and urinalysis before performing bronchoscopy at Visit 3
    - Visit 3 bronchoscopy should be conducted between 1 and 14 days after a combined Visit 1 and Visit 2
- Combining Visit 2 and Visit 3
  - Visit 2 and Visit 3 (bronchoscopy) may be performed in one day.
    - Typically Visit 2 would be performed in the morning. Then Visit 3, bronchoscopy, would be conducted in the afternoon. To conduct the bronchoscopy participant must have abstained from eating or drinking for at least 6 hours before the procedure
    - If Visit 2 and Visit 3 are combined, Visit 1 must be conducted on a separate day

Phone visit 1

- Clinics should contact participant within 7 days to conduct phone visit 1 if possible. The phone call is designed to monitor recovery and possible adverse events from the bronchoscopy

Phone visit 2

- If phone visit 1 was not conducted within 7 days, combine phone visits 1 and 2 and complete both the P1 and P2 forms

3. Clinic Certification

3.1 Clinic center and staff certification 3-2
3.2 Data system certification 3-4
3.3 Consent statement checklist 3-5
3.4 Clinic and staff certification checklist 3-8
3.5 Study Supplies 3-9

3.1 Clinic center and staff certification

Purpose

- Provide confirmation and documentation that a clinic has obtained the required approvals, facilities, equipment, personnel, and training necessary to conduct Broccoli Sprout Extracts Trial (BEST)
- Ensure consistent conduct of study procedures over time within and across clinics so that findings from all clinics are comparable

When

- Before a BEST clinical center may recruit participants and conduct study procedures for the Broccoli Sprout Extracts Trial (BEST)
- Prior to receiving study drug

Tasks

Clinic center certification

- Acquisition of approval of protocol and consents from the IRB
- Submission of a copy of IRB's notice of protocol approval and a copy of each approved consent statement (with the stamp of approval from the clinical center's IRB, if applicable) to the Data Coordinating Center
- Review of BEST protocol and procedures
- Arrangements for facilities, equipment, and supplies that are needed to conduct BEST procedures
  - ☐ Secure area for drug storage in -20C/-70C
  - ☐ Private area to conduct health interviews, assess eligibility, and provide patient education
  - ☐ Secure area for storage of study supplies, documents, and forms
  - ☐ Facilities for disposal of unused drug supplies
  - ☐ Equipment to perform Spirometry, lung volumes and diffusion capacity
  - ☐ Equipment to measure height and weight
  - ☐ Pregnancy test kits
  - ☐ Laboratory equipment for sample processing (See section 3.5 for complete list of lab supplies)
  - ☐ Computer requirements for data entry
    - Microsoft Windows
    - Microsoft Internet Explorer (6.0 or above)
    - Adobe Acrobat Reader (available free from www.adobe.com)
    - printer
- Completion of the Clinical Center Certification form (CC) by the Lead coordinator
- Submission of a copy of reference lab values from your local lab.

NOTE: Receipt of a notification of clinical center and staff certification from the Data Coordinating Center (via e-mail) is required. Clinics cannot perform screening visits (v1) until written notification of certification is received

Requirements for all staff members

- Acquiring Personal Identification Number (PIN) to complete study forms and/or enter data
- Completion of Knowledge Assessments online. To access General Knowledge Assessment (GEN) log onto BEST website home page
  - Click on Certification; Knowledge Assessments
  - Follow instructions
  - 80% correct response is required to pass
  - When test is "passed", print out results and submit to DCC along with other relevant certification materials
  - Can be repeated unlimited number of times and is open book
- Attendance by key study personnel at 22 February 2010 training meeting or other training authorized by the Data Coordinating Center (DCC)
- Training of all personnel who did not attend formal BEST training meeting in study procedures and form completion, as well as Good Clinical Practice and research integrity by the Lead coordinator or the DCC
- Completing a Personnel Assurance Statement (PA)

Additional requirements for BEST Center Director

- Sign off on Clinical Center Certification (CC) form completed by Lead Coordinator

Additional Lead coordinator certification requirements

- Entering names and contact information of all new center staff members including laboratory personnel involved with specimen processing, into the online BEST Directory (Lead Coordinator only)
- Completion of Data System Operator requirements (see data system operator requirements below)

Additional Data System Operator requirements

- Completion of the certification data system (online, under Data System) by completing BEST data entry tutorial for staff members who will be entering data into the BEST database.
- Completion of Data System Operator Knowledge Assessment (DSO) (online, under Certification Materials)

Forms (abbreviation)

- Clinical Center Certification (CC)
- BEST Personnel Assurance Statement (PA)

3.2 Data system certification

Access to the BEST data system will be restricted to clinical center staff that are certified for data entry. Each clinical center will be required to have at least one person certified for data entry, although having more than one person certified is advised. It is recommended that the lead coordinator be data entry certified since data entry certification is required to enter the Randomization (RZ) form.

The tasks for data system certification are as follows:

- Review the protocol and data forms
- Have Lead Coordinator obtain PIN# from DCC
- Complete BEST Personnel Assurance Statement (PA)
- Complete General (GEN) and Data System Operator (DSO) Knowledge Assessments online and print out results
- Log onto BEST datasystem using PIN and password (see written directions below). If staff member has not previously created a unique password, use the default password "changeme" The system will instruct the user to create a unique password the first time that user logs in to the data system. From that time forward, only the user's unique passcode will work.

  - Under Data System, click on Certification Data System
  - Follow instructions including entry of the practice PF, and BH forms
- Submit (fax) printouts from data entry "test" along with the BEST GEN, DSO and PA, and other certification materials, if not previously submitted, to the certification coordinator at the Data Coordinating Center
- Receive email verification from Data Coordinating Center of certification and activation for BEST data entry

The data system will be accessed via the internet using Microsoft Internet Explorer (version 5.0 or higher) on a PC running Microsoft Windows (95/98/ME/NT/2000/XP). The data system is available from the BEST web page at http://www.besttrial.org. Access to any part of the BEST data system requires a PIN, password, and activation by the DCC.

Each attempt to access any part of the data system will lead the user to a login screen, requiring a clinic ID, PIN, and password. The "TEST" clinic is available to all users for practice with the data system. For submitting forms for data entry certification, the "Certification" link on the data system page should be used, (not the "TEST" login.) After a staff member becomes certified, his or her PIN will be activated for data entry.

3.3 Consent statement checklist

Clinic: _____ Reviewer: _____ Date of review: _____

BEST CONSENT CHECKLIST **Page 1 of 3**

The DCC is required to review each center's consent form for completeness. To be certified, the following items must be included in each center's BEST consent form in addition to statements required by your local IRB. **Review your consent form for items listed below <u>before</u> submitting to your local IRB**. If you are unclear as to the inclusion of certain items in your consent, you may submit this completed checklist and your consent to the DCC for review prior to submission to your IRB.
NOTE - For BEST certification, this completed checklist (noting the location in the consent where statement is found) must be submitted to the DCC along with your IRB-approved consent.

Record the page where item is found in your consent (in left-hand space below) and highlight the statement in your consent.

_____ 1. Letter or stamp of approval from IRB with date approved and expiration of approval

_____ 2. Full name of trial – Broccoli Sprout Extracts Treatment Trial (BEST)

_____ 3. Sources of funding/sponsorship – NHLBI of National Institutes of Health (NIH)

_____ 4. Participant encouraged to read the consent form carefully and to ask questions

_____ 5. No access to certain medical information and test results during study, but can be obtained in medical emergency

_____ 6. Participation in this research study not meant to replace usual care for COPD.

_____ 7. You should inform your COPD physician (if applicable) of participation in the study

_____ 8. Purpose of trial: To determine if taking Sulforaphane can reduce lung inflammation and increase ability to resist infections in people with COPD.

_____ 9. This is not a treatment study

_____ 10. General description of COPD

_____ 11. List of inclusion criteria
   a. Mild to moderate COPD
   b. Males and females, 40 years and above

_____ 12. Approximately 90 people will participate

_____ 13. Length of participation about 6-8 weeks

_____ 14. General description of Sulforaphane and placebo

_____ 15. General description of randomization

_____ 16. Study treatment masked to participant and clinic staff. Study doctor can find out treatment in an emergency

_____ 17. Description of 5 clinic visits and 3 phone calls – questionnaires and procedures (bronchoscopy, spirometry, DLCO, physical exam, nasal brushing, pregnancy screening, questionnaires, and blood samples).

_____ 18. Biological samples collected for this research to be kept for future study

_____ 19. Risks/Discomforts
Possible side effects of study drug (Sulforaphane)
- Bad taste, poor appetite, upset stomach, and unknown side effects

_____ 20. Possible side effects of study procedures
a. Spirometry – chest soreness or dizziness
b. Albuterol – nervousness/shakiness, rapid heartbeat, headaches; rarely arrhythmias, low potassium
c. Bronchoscopy – pain, cough, discomfort, fever; rarely difficulty breathing
d. Nasal brush – burning sensation, watering eyes, nosebleed
e. Anesthetic medications – dizziness, headache, unusually slow or fast heartbeat, confusion or excessive tiredness
f. Lidocaine – stinging, burning, tenderness
g. Blood draw – pain, bruising, lightheadedness

_____21. Cannot participate if pregnant or breastfeeding. If participant becomes pregnant during the study, they must inform study doctor immediately

_____22. Females who have reached menarche and not reached menopause will have pregnancy tests at screening

23. Benefits

_____ a. No guaranteed health benefit for participating

_____ b. Results of the research may benefit others

24. Payment/cost for participation

_____ a. No cost to participant

_____ b. Some financial compensation

25. Voluntary nature of participation

_____ a. If decide not to participate, access to medical care at (site) will not be affected

_____ b. Can agree to be in the study now and change mind later. Information collected up to that point cannot be retracted

_____26. Participation may be discontinued early by study physician for various reasons as listed

27. HIPAA/Confidentiality – how personal data is handled

_____ a. Information collected includes contact information and personal health information

_____ b. Any information with participant's name to be kept in a locked cabinet.

_____ c. Unique number and special code will be used in place of your name

_____ d. Organizations such as governmental agencies, participating doctors and staff, processing labs, and sponsors may see your health information

_____ e. Can cancel permission to use and disclose information. If withdraw permission to use information, your part in study will end. Cancellation will not affect data already collected

_____28. Compensation for injury – Johns Hopkins, and the Federal government noted as not responsible. Participant and his/her insurance company are responsible for costs due to injury

_____29. Johns Hopkins University has a patent on the method for producing Sulforaphane from Broccoli Sprouts and for other uses of sulforaphane and may receive payments for production and use of sulforaphane.

_____30. Contact person – at least one name and number for questions, problems, or reactions to study drug (i.e., PI or coordinator)

_____31. If you join this study you will not own the data or specimens given by you to the investigator for this research

_____32. Researchers may ask to see your health care records

_____33. Signing of consent – spaces for signature and date for participant, and/or the study investigator or person obtaining consent

3.4 Clinic and staff certification checklist

Clinic: _____

BEST Certification Checklist

The following documents and procedures need to be completed by each clinic. **Please check off items as applicable, and submit to the DCC with your certification package, as the cover page.**

Center Certification form

□ Clinic Certification (CC) form (completed by Lead Coordinator only and signed by Principal Investigator)

Study documents

□ IRB notice of approval of BEST protocol
□ IRB approved consent statement with IRB stamp or notice of approval with consent checklist" items highlighted
□ Consent checklist denoting pages where items exist in the center's approved consent
□ HIPAA statement or other documents, if not included in main consent
□ Reference Laboratory values for your local lab

All investigators/staff members requirements

□ General BEST Knowledge Assessment (GEN) – printout from online test
□ BEST Personnel Assurance Statement (PA)

Additional Center Director/Co- PI at satellite requirements

□ Sign Clinical Center Certification (CC) form

Additional Lead Coordinator requirements

□ Complete Clinical Center Certification (CC) form
□ Enter all new staff members seeking certification into the ACRC online directory
□ Complete all certification procedures required for data system operator
- online Data system knowledge assessment (DSO).
- Submit printout from online BEST Certification Data System test exercise (link for Certification Data System on the BEST web page under Data System) and submit the confirmation pages.

3.5 Study Supplies

Data Coordinating Center will distribute the following at the beginning of the study and as needed:

- Clinic Supplies
  - Sealed treatment unmasking envelopes

- Participant supplies
  - Insulated lunch bags with gel packs
  - Aero chamber
  - BEST Wallet Cards
  - Cruciferous vegetables list

- Questionnaires
  - Forms and other questionnaires will be posted on the BEST website (http://www.besttrial.org/)

- Specimen collection supplies
  - BAL lavage
    - Cryovials, orange cap
    - Slides
    - 120 mL specimen jar (orange top)
    - 2 mL micro centrifuge tubes

  - Bronchial and nasal brushings
    - Nasal brushes
    - Cryovials, orange cap
    - 2 ml micro centrifuge tubes
    - 50 mL falcom tube (blue top)
    - 50 mL falcom tube (red top)

  - EBC
    - RTube™ (disposable plastic collection tube and mouth piece) for EBC collection
    - 2.0mL conical cryovials, white top
    - Cooling sleeve with insulated cover (2)
    - Plunger
    - Freezer bags for cooling sleeve
    - Transfer pipettes

  - Blood – PBMCs
    - 8mL CPT vacutainer (red/gray top)
    - 2 mL micro centrifuge tubes
    - Cryovials, orange cap

  - Blood – Plasma
    - 6mL vacutainer (green top)
    - Cryovials, orange cap

- Blood – Serum
  - 5mL SST vacutainer (gold closure)
  - Cryovials, orange cap

- Labels
  - Pre-printed Vacutainer Label Sheet
  - Pre-printed Cryovial Label Sheet
  - Pre-printed EBC Label Sheet
  - Pre-printed Participant ID Label Sheet

- BEST shipping supplies
  - Cardbox specimen storage box with 9×9 insert
  - Slide mailers
  - Styrofoam shippers
  - Plastic biohazard specimen bags
  - Absorbent sheets
  - Dry ice label (UN 1845)
  - UN3373 label

Ordering additional supplies from Data Coordinating Center

- Order supplies as needed
- Complete BEST Supply order (BO) form
- Requests should include number of items and date needed
- Fax to DCC (Fax number 443)

Clinical Center will provide:

- Refer to section 5.10 for the list of laboratory supplies and equipment
- Other supplies
  - Blood collection supplies (needles, tourniquet, etc)
  - Scotch tape
  - FedEx mailing labels
  - Packing material (bubble wrap, “peanuts”)
  - Pregnancy test kits
  - Nose clips for spirometry and EBC collection
  - Insulated gloves for handling aluminum cooling sleeve (EBC) and/or dry ice
  - Spirometry mouthpieces, if applicable
  - Albuterol and spacer for bronchodilator testing

Forms (abbreviation)

- BEST Supply order form (BO)

4. Procedures

4.1 Lung Function Test
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4.1.1 Spirometry

Purpose

- Measure FEV1 (the amount of air expired in the first second during a forced expiratory maneuver) and FVC (forced vital capacity).

When

- Visits 1, 2, and 4

Whom

- All Spirometry testers are not required to be certified by the DCC but should either be certified pulmonary function technologists (National Board of Respiratory Care) or receive sufficient instruction from qualified instructors to perform the procedure according to acceptable standards.

Equipment

- Local hospital spirometry equipment will be used
- Equipment and procedures should be based on the ATS recommendations for accuracy and precision

Predicted values

- Predicted values should be calculated according to the published predicted values (Hankinson et al). Use values listed in Table 4.1.1.1 or calculate online using the BEST pulmonary function calculator. To access the online calculator go to the BESTT website: http://www.besttrial.org and follow the link to the BEST data system page and then to the calculator

Participant preparation

- **For visit 1 only**

If lung function criteria demonstrated within the past 6 months (historical), no spirometry procedure is requiredfor eligibility.

  - If historical lung function criteria not demonstrated, conduct pre and post spirometry at Visit 1
  - Lung function criteria : FEV_1 40-80% and FEV/FVC <0.7 predicted within past 6 months

- Spirometry test should be performed at least :
  - 4 hours after last dose of short-acting bronchodilator (eg, Atrovent, Combivent, albuterol, ipratropium, Ventolin, Proventil)
  - 12 hours after the last dose of long-acting bronchodilator (eg, Serevent, Advair, salmeterol, theophylline, Dulera, Symbicort)
  - 24 hours after ultra long acting bronchodilator (eg, tiotropium, Spiriva)
- Restrictive clothing should be loosened
- Participant should be seated with feet flat on floor, and wearing nose clips.

Testing procedure

- Perform test until acceptability and reproducibility criteria according to ATS/ERS task force guidelines are met.

- The largest acceptable FEV and FVC should be recorded on the data collection form. These do not have to be taken from the same maneuver.
  - Calculate FEV percent predicted value:
    Use BEST online calculator from BEST website: www.besttrial.org. Follow link to data system page and then to the BEST calculator

    OR
  - Manually calculate using predicted FEV value per Hankinson (formula in Table 4.1.1.2.) and the following formula: Percent predicted = FEV / predicted FEV x 100

Note: If your spirometry system uses Hankinson you must make sure the values obtained from your system are the same as those calculated by the BEST data system. Rounding conventions may be different causing discrepancies

Pre- and post-bronchodilator procedures

- Perform pre-bronchodilator spirometry on patient
  - Spirometry is performed before any bronchodilators are used
  - Short-term bronchodilator should be held for 4 hours prior to testing
  - Long-term bronchodilators should be held for 12 hours prior to testing
  - Record results on the Screening (SC) form for Visit 1 and Pulmonary Function Testing (PF) form for Visits 2 and 4

- Administer bronchodilator
  - Administer 2 puffs of metered dose inhaler (MDI) albuterol
  - Wait 15 - 30 minutes after administering bronchodilator before retesting

- Perform post-bronchodilator spirometry
  - Record results on Pulmonary Function Testing (PF) form

4.1.1.1. Table of Pulmonary Function Predicted Values

The general form of the prediction equations is: $PFT = Z + A(Age) + B(Age^2) + C(Height^2)$

PFT	Ethnicity	Gender	Z	A	B	C
FVC	Caucasian adults	Male	-.1933	.00064	-.000269	.00018642
		Female	-.3560	.01870	-.000382	.00014815
	African-American adults	Male	-.1517	-.01821	----------	.00016643
		Female	-.3039	.00536	-.000265	.00013606
	Mexican-American adults	Male	.2376	-.00891	-.000182	.00017823
		Female	.1210	.00307	-.000237	.00014246
	Caucasian child	Male	-.2584	-.20415	.010133	.00018642
		Female	-1.2082	.05916	----------	.00014815
	African-American child	Male	-.4971	-.15497	.007701	.00016643
		Female	-.6166	-.04687	.003602	.00013606
	Mexican-American child	Male	-.7571	-.09520	.006619	.00017823
		Female	-1.2507	.07501	----------	.00014246
FEV_1	Caucasian adults	Male	.5536	-.01303	-.000172	.00014098
		Female	.4333	-.00361	-.000194	.00011496
	African-American adults	Male	.3411	-.02309	----------	.00013194
		Female	.3433	-.01283	-.000097	.00010846
	Mexican-American adults	Male	.6306	-.02928	----------	.00015104
		Female	.4529	-.01178	-.000113	.00012154
	Caucasian child	Male	-.7453	-.04106	.004477	.00014098
		Female	-.8710	.06537	----------	.00011496
	African-American child	Male	-.7048	-.05711	.004316	.00013194
		Female	-.9630	.05799	----------	.00010846
	Mexican-American child	Male	-.8218	-.04248	.004291	.00015104
		Female	-.9641	-.06490	----------	.00012154

Age is in years at last birthday
Height is standing height in cm
PFT predicted values are in liters
Predicted values for Latinos will be as for Mexican-Americans
Predicted values for other ethnic groups will be as for Caucasians
Participant's ethnic identity is self-defined
Adult $\geq$ 20 years old for males and $\geq$ 18 years old for females

Participant specific predicted values may be obtained from the data system following the initial screening spirometry and printed as appropriate for the participant's chart or file.

4.1.2. Lung volumes

Purpose

- Measure FRC (functional residual capacity), RV (residual volume), SVC (slow vital capacity), and TLC (total lung capacity)

When

- Visits 2 and 4

Whom

- All personnel performing lung volume procedures are not required to be certified by the DCC but should either be certified pulmonary function technologists (National Board of Respiratory Care) or receive sufficient instruction from qualified instructors to perform the procedure according to acceptable standards.

Equipment

- Local hospital pulmonary function laboratory equipment will be used
- Helium dilution or Plethysmography methods will be used

Testing procedures

- Lung volumes should be measured in accordance with local hospital procedures

Forms (abbreviation)

- Pulmonary Function Testing (PF)

4.1.3. Carbon Monoxide Diffusing Capacity (DLCO)

Purpose

- Determination of the carbon monoxide uptake in the lung (DLCO) using the single-breath method

When

- Visits 2 and 4

Whom

- All personnel performing DLCO are not required to be certified by the DCC but should either be certified pulmonary function technologists (National Board of Respiratory Care) or receive sufficient instruction from qualified instructors to perform the procedure according to acceptable standards.

Equipment

- Local hospital pulmonary function laboratory equipment will be used

Testing procedures

- DLCO should be measured in accordance with local hospital procedures

Forms (abbreviation)

- Pulmonary Function Testing (PF)

4.2.1 Nasal Brushings

When

- Visits 3 and 5
- For participant convenience, nasal brushings are obtained at the time of the bronchoscopic procedure, but may also be obtained at a separate visit.

Whom

- The operator is not required to be certified by the DCC but should be a physician trained and certified to perform the procedure or under the direct supervision of a trained and certified physician.

Order of procedure

- This procedure should be performed prior to the bronchoscopy to coordinate the procedure with the nasal anesthesia and sedation used in the bronchoscopy.

Supplies

- **Supplied by DCC**
  - Nasal cytology brush (Hobbs Medical Cat. No: 4290)
  - 50 mL falcon tube (Blue top)
  - Preprinted label

- **Supplied by clinic**
  - Cotton swabs
  - Viscous Lidocaine and/or 2% topical lidocaine
  - PBS, 5mL

Conduct of the procedure

- The falcon tube (blue top) is filled with 3-5 mL PBS solution and affixed with a preprinted label
- The nasal passage is anesthetized in accordance with operator preference using either sterile swabs with viscous lidocaine, instillation of topical lidocaine, or both.
- The procedure may be done either before or during induction of sedation.
- Either nostril may be selected for the procedure, depending upon the preference of the operator. If the patient reports a recent nosebleed from one nostril, that nostril should be avoided.
- The cytology brush is gently inserted into the nose approximately 3-4 cm and is gently rubbed back and forth 3 or 4 times with a gentle twirling motion along the mucosa of the inferior turbinate.
- Care should be taken not to abrade the mucosal in the anterior septal cartilage which has a tendency to bleed.

- The brush is withdrawn and washed in the falcom tube containing 3-5 mL of PBS with a shaking and flicking motion. The procedure is repeated for a total of 3-4 collections.
- The procedure may induce a slight abrasion of the nasal mucosa, but if frank bleeding occurs the procedure should be terminated.
- Nasal bleeding can be usually stopped by pinching the nostril for 5-10 minutes or instillation of oxymetazoline (Afrin®) nasal spray.

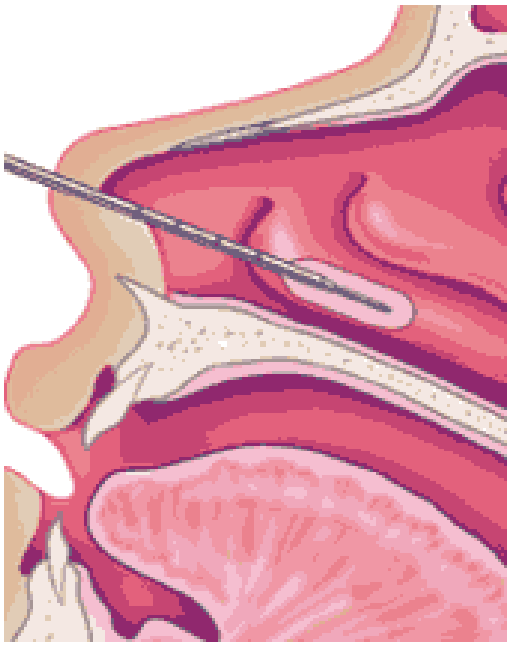

Figure showing the proper area for brushing along the medial nasal mucosa on the surface of the inferior nasal turbinate. The brush should be inserted perpendicular to the facial plane in the same trajectory as the bronchoscope is inserted.

4.2.2 Fiberoptic Bronchoscopy with Endobronchial brushings and Bronchoalveolar Lavage

Purpose:

- The objectives of the bronchoscopic procedure is to secure specimens of lung cells that will be assayed for evidence of NRF2 activity and that will be used for ancillary studies of bacterial clearance and corticosteroid sensitivity.

When

- Visits 3 and 5

Whom

- The operator is not required to be certified by the DCC but should be a physician trained and certified to perform the procedure or under the direct supervision of a trained and certified physician.

Forms and Supplies

- **Forms**
  Bronchoalveolar lavage/Bronchial brushings/Nasal brushings form (BL)

- **Supplies**

  Supplied by DCC
  - 50 mL falcon tube (Red top) for brushings
  - 120 mL specimen jar (orange top) for lavage
  - Preprinted labels

  Supplied by clinic
  - 1 or 2 bronchoscopes
  - 1 endobronchial cytology brush
  - 300 mL room-temperature sterile normal saline
  - 50 mL syringes
  - 1% or 2% topical lidocaine
  - Lidocaine topical gel
  - Sedative medication per local procedures and practice
  - PBS, 5 mL

- **Facilities**
  Should be conducted in a medically safe environment with properly trained personnel, proper monitoring and infection control procedures, such as a JCAHO-approved facility.

Scheduling

- Before scheduling the first of the two bronchoscopy procedures, it should be established that:
  - Patient has signed IRB-approved consent for the BEST research protocol,
  - Has met the inclusion criteria for the study.
  - The benefits and risks of the procedure have been explained to the participant and patient has given consent for the procedure.
- The target windows for scheduling the bronchoscopy is 3 weeks after first visit (on the day of randomization), for the first procedure and 3 to 4 weeks after first bronchoscopy, for the second procedure.

Patient preparation

- The participant should be interviewed prior to the procedure to ensure that there are no contraindications to performing the bronchoscopy such as
  - recent acute or serious illness
  - anticoagulation or bleeding disorder
  - fever
  - oxygen desaturation on room air
  - allergy to local anesthetics or sedation
  - food intake within 6 hours.
- Patients should be instructed to take their usual morning oral medications with a small sip of water.
- Patients should also have a companion or reliable escorted means of transportation home after the procedure so that they can safely return home after the procedure.
- The patient should be informed that the procedure is being performed for research purposes and is not for clinical care.
- The details of the procedure should be explained to the participant and consent obtained in accordance with local hospital or clinic practices and procedures.

Risks related to the procedure:

- allergic or adverse reaction to sedation or local anesthesia
- nose-bleed,
- sore-throat,
- coughing,
- chest soreness from coughing
- fever
- infection.

Conduct of the procedure

- **Sedation**
  - Before sedation, a "time-out" should be performed to verify that the patient is correctly identified and is undergoing a bronchoscopy.
  - The bronchoscopy insertion orifice should be ascertained (right or left nostril or mouth) and local anesthetic applied in accordance with local practice.
  - The patient is moderately sedated during monitoring of vital signs and oxygenation according to local procedures.
  - Typically a benzodiazepine (e.g midazolam) and a narcotic (e.g. fentanyl) are used.
  - The additional use of atropine or sedative antihistamines (e.g. diphenhydramine or phenergan) are optional.

- **Method**
  - The bronchoscope is inserted and the upper airway is anesthetized with 1-2 mL aliquots of lidocaine 1% or 2% solution.
  - If using a double scope method the initial bronchoscope can be replaced with a second bronchoscope that does not have a contaminated inner channel if samples of the lower airway are to be collected for culture.
  - The lower airways are anesthetized with aliquots of lidocaine to diminish or prevent coughing.
  - The total amount of lidocaine instilled should be monitored and typically *no more than 500 mg* should be administered in a single procedure.
  - In order to minimize the use of lidocaine, the inspection of the left lung airways should be brief or limited.

- **Collection of specimens**

  The bronchoscope is advanced into the right mainstem bronchus and the following specimens are collected in the preferred order below:

  Bronchial Cytology brushings
  Bronchoalveolar lavage (BAL)

Cytology Brushings

- The falcon tube (red top) is filled with 3-5 mL PBS solution and affixed with a preprinted label
- A total of 10 cytology brushings are collected under direct visualization from 3-5 sites in the proximal airways.
- The rationale for performing the brushings first is that it is usually well tolerated in terms of coughing and oxygenation and it will be possible to obtain these specimens in nearly all patients regardless of BAL returns.
- Dependent airways that will not be used for subsequent BAL are preferred so that any minor bleeding will not contaminate the BAL return.

- The most approachable dependent airways are the
  posterior segment of the right upper lobe,
  superior segment of the right lower lobe,
  posterior basilar segment of the right lower lobe
  right mainstem bronchus.
- Brushings may also be taken from the carina.
- Each brushing specimen is acquired by vigorously moving the brush about 5-10 mm, 6-8 times.
- It is common that the brushings will cause abrasion of the airway surface. However, if there appears to be frank bleeding, as occurs in some patients with friable mucosa, the vigor of the brushings should be adjusted.
- Areas of airways that are coated with visible mucus should be avoided to avoid clogging of the cytology brush.
- The cytology brush is withdrawn into the protective plastic sheath and withdrawn. After each brushing, the cytology brush is washed with a "flicking" motion in a red top falcon tube containing 3-5 mL PBS.
- All of the specimens are washed into the same pre-labeled red top falcon tube, for later processing.

Bronchoalveolar lavage (BAL)

- Performed by instillation of 2-50 mL aliquots in each of three regions of the lung in the following preferred order:
  Right middle lobe either segment,
  Right upper lobe anterior segment,
  Right lower lobe anterior segment.
- Patients with COPD have smaller or sometimes absent BAL returns than other patients because of trapping of the fluid in emphysematous regions or behind closed or obstructed airways.
- Return of BAL fluid can sometimes be facilitated by ensuring that the return suction is not too high (< 100 cm H2O), by turning the patient with the right lung superior, or by asking the patient to take a deep breath and cough.
- The quantity of return from each of the three segments is noted and recorded, and all of the three BAL specimens are pooled in a 120 mL specimen jar (orange top) and stored on ice for immediate processing.

Ancillary studies

- For ancillary studies of lower airway microbiology, a modified procedure is used. So that accurate microbiology cultures and PCR are obtained from the BAL.
- The channels of the collecting bronchoscope should not be contaminated with lidocaine or upper airway secretions. Accordingly, a modified "double bronchoscope" procedure should be used.

- In this procedure, the first bronchoscope is used for application of local anesthesia to the upper and lower airways and to obtain the airway brushings.
- Following this, a second sterile bronchoscope is inserted into the lower airways for collection of the BAL.
- To avoid inadvertent contamination, the suction should not be connected to the bronchoscope until the BAL is to be collected, and no instruments or drugs should be instilled through this scope prior to the BAL.

Definition of a successful procedure.

- Participants will be randomized to treatment if they complete a successful initial bronchoscopy.
- It is anticipated that some procedures will not successfully provide all of the samples if the procedure needs to be terminated early because of patient safety or comfort concerns, or if the yield of BAL fluid is inadequate.
- Even small returns of BAL fluid of 5-6 mL may be adequate for analysis of some of the study outcomes if the cellularity of the specimens is sufficient. Thus, no specimens should be discarded.
- However, we are considering a bronchoscopy procedure to be successfully completed for the purpose of randomization if the patient underwent sedation for the procedure and at least some of the specimens such as nasal or bronchial brushings were obtained, and the clinic physician does not conclude that it would be unsafe for the patient to have the second procedure.

Completion of procedure

- The BAL form is completed which records the results of the BAL.
- The research coordinator should be notified that the procedure is completed.
- If the participant is undertaking the first procedure, the research coordinator will need to enter the data system to obtain a randomization code and provide the patient with study drug.
- If the patient has a post-bronchoscopy fever, the use of anti-pyretics are advised.

Post bronchoscopy care.

- The patient should be monitored in supervised environment until they have recovered from sedation, and vital signs and oxygenation are stable.
- For first procedures, the patient should not leave the recovery area to return home until the research coordinator is able to provide the patient with study drug or suitable arrangements are made for such
- The patient should be instructed not to take any food or liquid by mouth for at least two hours after the procedure or until the gag-reflex has returned.
- The patient should be notified to contact the clinic or study physician if any symptoms of concern are noted after the procedure.

4.3 Exhaled Breath Condensate (EBC)

Purpose

To collect liquid condensate from exhaled breath to evaluate measures of oxidative stress

When

- V2 and V4
- Before pulmonary function testing

Supplies

Provided by DCC

- RTubes (disposable plastic collection tube and mouthpiece)
- Aluminum cooling sleeve (2)
- Blue insulated cover for cooling sleeve (2)
- Evacuation plunger
- Transfer pipettes
- 2.0mL conical cryovials, white top
- Cryovial labels
- Cryovial box for storing specimens in freezer and shipping

Provided by Clinic

- Insulated freezer gloves
- Cooler and ice as needed for temporary storage of cooling sleeves

Prepare

Participant - No food and beverages for one hour

Equipment

- Chill 2 aluminum cooling sleeves
  - Put aluminum cooling sleeve in freezer plastic bag to prevent moisture from freezing to inside of sleeve
  - Chill in -20^{o} C or -70^{o} C freezer for 1-2 hours or in a cooler with dry ice for 15 minutes
  - If collection site is not near freezer, transport cooling sleeve in a cooler filled with ice or frozen packets
- Check valve operation on RTube per instructions appended to this section
- Assemble cooling sleeve/RTube

- Orient RTube with valve pointing up, away from mouthpiece
- Put on insulated gloves and remove aluminum cooling sleeve from freezer
- Put blue insulation cover over cooling sleeve to protect hands
- Place insulated cooling sleeve over RTube

Collect

- Begin collection immediately after cooling sleeve and RTube are assembled
  - Instruct the participant to breathe through mouth. Can use nose clips
  - Participant is seated holding RTube upright (if necessary, provide a support for arm holding RTube)
  - Participant breathes normally in and out through the mouthpiece, but must be hard enough to hear breath flowing through the top of the tube
  - If you cannot hear breath coming out the top of RTube, the valve may be stuck. Have participant to blow hard to force valve open; if valve does not open, use another RTube. (Let the DCC know about the defective R-tubes.)
- Monitor participant
  - If participant gets tired, s/he may take short breaks
  - If an extended break is needed, it may be necessary to change to a fresh chilled cooling sleeve
  - Regardless of breaks, actual time breathing through RTube should add up to 7 and half minutes for each session and RTube must remain upright at all times.
- After about 7 and half minutes, replace cooling sleeve with one just out of freezer or cooler but keep using the same RTube
- Have participant continue with fresh chilled cooling sleeve; actual collection time should be about 15 minutes

Aliquot

Aliquot immediately after collection

- Complete labels and put on cryovials
- Remove mouthpiece from RTube; discard mouthpiece
- Use evacuation plunger to gather sample near top of collection tube
  - Orient collection tube with the arrow on side and exhalation valve inside tube pointing up
  - Place collection tube on evacuation plunger
  - Pull collection tube down over evacuation plunger; the exhalation valve inside of the tube will move up the tube collecting the sample near the top of the tube
- Transfer into 3 cryovials (1.5 μl in first 2 cryovials and remainder in 3rd)
- Place cryovials on ice; freeze at -70^{o} C until shipping

Clean plunger

- Wear gloves
- Firmly grip plastic collection tube and gently pull off plunger
- Dispose of plastic collection tube
- Wash plunger with soap and water or wipe down with alcohol
- Allow plunger to dry before next use

Forms (abbreviation)

Clinic Visit Form (CV)

5. Specimen processing and shipping

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5.1 General requirements

- Gloves must be worn at all times when handling specimens. This includes during removal of the rubber stopper from the blood tubes, centrifugation, pipetting, disposal of contaminated tubes, and clean up of any spills. Tubes, needles, and pipets must be properly disposed of in biohazard containers, in accordance with institutional requirements.

- Universal precautions and OSHA (Occupational Safety and Health Administration) and institutional requirements (http://www.osha.gov/SLTC/biologicalagents/index.html) should be followed, including gloves, eye protection or working in a biosafety cabinet for blood processing.

- For complete list of clinic supplies, please refer to section 5.11 for catalogue numbers

- General lab supplies and equipment for sample processing (supplied by clinic) include:
  - Pasteur pipette; Glass
  - 50ml conical centrifuge tubes
  - Sterile disposable pipettes (5, 10 and 25ml)
  - Pipette aid
  - Laminar flow hood
  - Hemocytometer
  - Pipettman
  - Multichannel Pipette (Optional)
  - Centrifuge with swing bucket rotor (Refrigerated and ambient temperature)
  - Centrifuge (for microcentrifuge tubes)
  - -80^0 C and -20^0 C freezer
  - CO_2 incubator
  - CO_2 cylinder
  - Ice machine
  - Small liquid nitrogen tank

- Shipping supplies include
  - Supplied by DCC
    - Styrofoam box with outer cardboard box
    - Plastic biohazard specimen bag
    - Absorbent sheet
    - Dry ice label
    - UN3373 label
  - Supplied by clinic
    - Dry ice
    - FedEx air bill

5.2 PBMCs separation

When

- Visits 2 and 4

Supplies

- Supplied by DCC
  - 8mL green/red top CPT™ vacutainers (2)
  - 2mL microcentrifuge tubes (3)
  - Labels
- Supplied by clinic
  - Lab equipment and supplies
  - PBS, 500ml
  - HBSS , 500ml
  - RPMI 1640 medium
  - RLT buffer
  - ACK lysing buffer (for lysing RBCs)
  - Trypan blue

Procedure

1. Collect blood via venipuncture directly into two CPT tube(s) (each CPT tube will hold ~8 ml blood).

2. After collection, store tube upright at room temperature until centrifugation. Blood samples should be centrifuged within 1 hour of blood collection for best results.

3. Remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times.

4. Centrifuge CPT tube(s) at 1800g (approximately 2800 rpm on a Sorvall RT6000 centrifuge) for 30 minutes at room temperature. Be sure that the tubes are not loaded in the outer positions of the carriers, as this may cause the rubber tops of the tubes to contact the rotor and come off. See that the tube is in the proper centrifuge carrier/adapter.

Note:

a) Excessive centrifuge speed (over 2000 RCF) may cause tube breakage and exposure to blood and possible injury. To calculate the correct centrifuge speed for a given RCF, use the following formula:

$$\text{RPM Speed Setting} = \text{Sqrt}\left[\frac{(\text{RCF}) \times (100{,}000)}{(1.12) \times (r)}\right]$$

Where *r* (expressed in centimeters) is the radial distance from the centrifuge center post to the tube bottom, when the tube is in the horizontal position and RCF is the desired centrifugal force, 1800 in this case.

b) Always balance the rotor properly. Use a precision scale. Always balance the tube. For swinging buckets, be sure the buckets are weighed with their caps in place, that the seals are intact and that the caps are secure. Be careful in the placement of tubes within a rotor to ensure proper balance - check the manufacturers guides for complex rotors that hold multiple tubes.

5. After centrifugation, bring the CPT tube(s) to a biological safety cabinet and carefully open the tops. Mononuclear cells and platelets will be in a whitish layer just under the plasma layer. Aspirate approximately half of the plasma without disturbing the cell layer. Collect cell layer with a Pasteur Pipette and transfer to a 50 mL size conical centrifuge tube with cap. Collect the cells immediately following centrifugation. Transfer the cell layer of the two CPT tubes into one 50 ml conical centrifuge tube.

6. Add PBS to bring volume to 15 mL. Cap tube. Mix cells by inverting tube 5 times. Centrifuge for 8 minutes at 500g or RCF (RCF: Relative Centrifugal Force). Aspirate as much supernatant as possible without disturbing cell pellet. If there are appreciable amount of RBCs in the PBMC pellet, carryout RBC lysis step (Refer note). Gently tap the tube and re-suspend cell pellet in 10 ml HBSS. Gently mix cell suspension by pipetting up and down 5-10 times. Remove 10 µl aliquot of cell suspension for counting using a hemocytometer.

Note:

a) RBC lysis: If there is appreciable amount of RBC contamination in the PBMC pellet, add 1ml of ACK lysing buffer and incubate for 5 minutes at room temperature. Dilute the ACK lysing solution by adding 9ml of RPMI 1640 medium and continue from step 7.

b) Cell count: To determine the number of cells present in the sample
   - Mix 10µl of resuspended fluid with 90µl of trypan blue.
   - Load 10µl of this mix into each chamber of a hemocytometer. Count four squares in each chamber. Maintain two counts, one viable, one non-viable.
   - Calculate and record cell concentration for every chamber, as follows: i. **(**Total cells counted/4**) x** 10 **x** 10000 = number of cells per ml
   - Calculate and record **total** cell number by multiplying "cells per ml" by the 10ml volume after resuspending in RPMI.
   - Cell viability will be recorded as % of viable cells: **(**viable cells/total cells**) x** 100%

7. Centrifuge for 10 minutes at 500g. Aspirate as much supernatant as possible without disturbing cell pellet. Gently tap the tube and re-suspend cell pellet in 6 ml of RPMI 1640 medium. Gently pipette up and down to suspend the cells.

8. Divide the cell suspension into three aliquots (minimum of 3 million cells each into aliquot 1 and aliquot 2 and remaining cells into aliquot 3; use preprinted labels) into 2 ml microcentrifuge tubes. Centrifuge Aliquot 1 and Aliquot 2 at 500g for 8 min. Aspirate as much supernatant as possible from each tube without disturbing the cell pellet. Add 0.6ml of RLT buffer to Aliquot 1 and mix. Snap freeze Aliquot 2 in liquid nitrogen. Store both of the tubes at -80C.

9. Use Aliquot 3 for functional assay. (Ancillary studies at SUNY and JHU laboratories, Please refer to section on Functional assay-5.8)

Note:
Temple University: Because there are no functional assays conducted, Centrifuge the aliquot 3 at 500g in a refrigerated centrifuge set at 4^0C for 8 minutes. Immediately, remove the supernatant as much as possible and snap freeze the sample in liquid nitrogen. Store at -80C and ship to JHU.

5.3 Serum separation

When

- Visits 2 and 4

Supplies

- Supplied by DCC
  - 5mL gold top serum vacutainers (2)
  - 2mL cryovials (4)
  - Labels
- Supplied by clinic
  - Lab equipment and supplies

Procedure

1. Collect blood in two yellow top, blood collection tubes (5ml SST™ Hemogard vacutainers).

   Note: Use yellow top (serum) tubes (silicon-coated serum separator tubes). These tubes, without additives, allow the red blood cells to form a clot. The clot also includes white blood cells, platelets etc. After centrifuging, the clot is at the bottom of the tube, and the serum is on top of the clot). The yellow top tubes do not have to be full to be used.

2. Filled two yellow top blood collection tubes should sit upright after the blood is drawn at room temperature for a minimum of 30 to a maximum of 60 minutes to allow the clot to form.

3. Centrifuge the blood sample at the end of the clotting time (30-60 minutes) in a horizontal rotor (swing-out head) for 20 minutes at 1100-1300g at room temperature. [Warning: Excessive centrifuge speed (over 2000g) may cause tube breakage and exposure to blood and possible injury].

4. While centrifugation, label 4 cryovials with preprinted labels labeled Aliquot 1, Aliquot 2, Aliquot 3 and Aliquot 4.

5. Use pipette to transfer the serum (Recommendation: do not pour!) from both the tubes into a 15 ml conical tube. . Mix gently by vortexing and pipette serum into the four labeled 2 ml cryovials (Do not fill above the 1.8 ml line). Screw on caps tightly. Store Aliquot 1 and Aliquot 2 for shipment to JHU for analysis. Store Aliquot 3 and Aliquot 4 locally. Store all the samples at -80^{0}C/-70^{0}C. This process should be completed immediately after centrifugation.

6. All specimens should remain at -80ºC prior to shipping. The samples should not be thawed prior to shipping.

5.4 Plasma separation

When

- Visits 2 and 4

Supplies

- Supplied by DCC
  - 6mL green top plasma vacutainers (2)
  - 2mL cryovials (4)
  - Labels
- Supplied by clinic
  - Lab equipment and supplies

Procedure

1. Collect blood into two Sodium heparin coated 6ml tubes (BD Hemogard Vacutainer, green top)

2. After collection, gently mix the blood by inverting the tube 8 to 10 times. Store vacutainer tubes upright at 4ºC until centrifugation. Blood samples should be centrifuged within 1 h of blood collection.

3. Centrifuge blood samples in a horizontal rotor (swing-out head) for 20 minutes at 1200 g at room temperature. Excessive centrifuge speed (over 2000 g) may cause tube breakage and exposure to blood and possible injury.

4. After centrifugation, plasma layer will be at the top of the tube. Mononuclear cells and platelets will be in a whitish layer, called the "buffy coat", just under the plasma and above the red blood cells.

5. While centrifugation, label 4 cryovials with pre-printed labels for Aliquots 1-4.

6. Use pipette to transfer the plasma (Recommendation: do not pour!) without disturbing the buffy coat from both the tubes into 15 ml conical tube. Mix gently by vortexing and pipette plasma into the four labeled cryovials (do not fill above the 1.8 mL mark). Close the caps on the vials tightly. Save Aliquot 1, Aliquot 2 and Aliquot 3 cryovials for shipment to JHU. Store Aliquot 4 locally. Store all the samples at -80^0C. This process should be completed immediately after centrifugation.

7. All specimens should remain at -80ºC prior to shipping. The samples should not be thawed prior to shipping.

5.5 Collection of cells after nasal brushing

When

- Visits 3 and 5

Supplies

- Supplied by DCC
  - Nasal brush (1)
  - 2mL microcentrifuge tube (1)
  - 50 mL falcon tube, sterile (blue top)
  - Labels
- Supplied by clinic
  - Lab equipment and supplies
  - PBS, 500ml
  - RLT buffer

Procedure

1. Collect the cells from the nasal epithelium and introduce the nasal brush into 50 ml falcon tube (blue top) containing 3-5 ml PBS. Vigorously shake the brush to release the cells. Transfer the tube to the lab on ice within 1 hour.

2. Gently vortex and aliquot 10 ul of cell suspension for cell counting by hemocytometer. Record the total cell count (eg. 5 million) on the BAL Processing (BB) form.

3. Centrifuge for 8 min at RT at 2050xg. Remove supernatant as much as possible and immediately add 0.6 ml of RLT buffer. Mix it well by pipetting up and down (at least 5 times).

4. Prepare one 2mL microcentrifuge tube (labeled with preprinted Aliquot 1 label) Transfer the RLT buffer into 2mL microcentrifuge tube and store in the participant's JHU sample storage box. Store at -80^0C.

5.6 Collection of cells after bronchial brushing

When

- Visits 3 and 5

Supplies

- Supplied by DCC
  - 2mL microcentifuge tube (1)
  - 50 mL falcon tube, sterile (red top)
  - Labels
- Supplied by clinic
  - Lab equipment and supplies
  - PBS, 500ml
  - RLT buffer

Procedure

1. After the bronchial brushing procedure, rinse the brush vigorously in 50 ml falcon tube (red top) containing 3-5mL PBS. Transfer the tube to the lab on ice within 1 hour

2. Gently vortex the 50 ml falcon tube containing to release the cells. Open the tube in the biological safety cabinet. Gently mix by pipetting up and down. Take aliquot of 10 ul for cell counting. Record the total cell count (for eg. 3 million) on the BAL Processing (BB) form.

3. Centrifuge the cell suspension at 2050xg for 8 min.

4. Remove supernatant as much as possible and immediately add 0.6 ml of RLT buffer. Mix gently with a 1 mL pipette and transfer the solution into labeled 2mL microcentrifuge tube (use pre-printed lables). Store the cryovial in the participant's JHU sample storage box at -80^0C.

5.7 Bronchoalveolar lavage processing and cell separation

When

- Visits 3 and 5

Supplies

- Supplied by DCC
  - 2mL cryovials
  - Slides (6)
  - 2mL microcentrifuge tubes
  - Labels
  - Slide box
- Supplied by clinic
  - Lab equipment and supplies
  - PBS, 500ml
  - RLT buffer
  - Sterile guaze
  - Rubber Cell Scraper
  - HBSS , 500ml
  - RPMI with phenol Red
  - RPMI without Phenol red
  - Fetal Bovine Serum, Heat Inactivated
  - EDTA solution
  - PBS, 500ml
  - RLT buffer
  - Tryphan blue – 50mL falcon tube
  - Differential Quik Stain Kit– 15mL falcon tube
  - CO_2 incubator– 15mL falcon tube
  - CO_2 cylinder
  - Acetone

Procedure

Note:

a) The following procedure is to be performed wearing laboratory coat, gloves, eye protection, and mask. Perform all open tube procedures in a biological safety cabinet (BSC) in a level 2 laboratory. Check, if Level 3 pathogens are suspected to be in the sample.

b) The bronchoalveolar lavage (BAL) fluid must be fresh and processed as soon as possible after collection (within 1 hour).

1. Label all the tubes needed for this procedure BEFORE the start of the protocol.

2. ICU Doc's will collect BAL fluid in two 120 mL specimen jars (orange top).

3. After collection place the specimen jars on ice immediately.

4. After the procedure, transport the specimens, on ice, back to the lab immediately for processing.

5. Pool the contents of the trap(s) of BAL fluid if necessary.

6. Filter the BAL fluid sample through sterile gauze into a sterile 50ml falcon tube to remove mucus plugs. Note the volume after filtering and record on the BAL Processing (BB) form. .

7. Centrifuge the sample at 500xg in a refrigerated centrifuge set at 4 degrees for 10 minutes.

8. Decant supernatant into a sterile 50ml Falcon tube. Make 8-10 aliquots of 1.8 mL volume into labeled cryovials using pre-printed labels. Save Aliquot 1-4 for shipment to JHU for analysis. Store at -80^{o}C. Store Aliquots 5-10 locally.

9. Gently tap the tube and re-suspend cell pellet in 10 ml of ice-cold Hanks Buffered Saline Solution (HBSS). Gently mix by pipetting up and down.

10. Centrifuge the remaining sample at 500xg in a refrigerated centrifuge set at 4^{o}C for 10 min. Discard supernatant.

11. Re-suspend cell pellet in 10ml of cold HBSS.

12. Repeat steps 9 and 11.

13. Re-suspend cell pellet in 10ml of RPMI media supplemented with 10% FBS.

14. Aliquot 10 µl of suspension for cell counting. Count the pellet cells in the 10 uL aliquot. Record the cell number in the BAL Processing (BB) form. Keep aside 0.6mL suspended pellet for 6 cytospin slides.* (See procedure for cytospin slides #23)

    Note: Determine the number of cells present in the sample:
    a. Mix 10µl of resuspended fluid with 90µl of trypan blue.
    b. Load 10µl of this mix into each chamber of a hemocytometer. Count four squares in each chamber. Maintain two counts, one viable, one non-viable.
    c. Calculate and record cell concentration for every chamber, as follows: i. **(**Total cells counted/4**) x** 10 **x** 10000 **=** number of cells per ml
    d. Calculate and record **total** cell number by multiplying "cells per ml" by the 10ml volume after resuspending in RPMI.
    e. Cell viability will be recorded as % of viable cells: **(**viable cells/total cells**) x** 100%

15. Transfer cells suspended in RPMI media to a sterile 10cm culture dish.

16. Incubate at 37^{o}C with 5% CO2 for 1 hour to let macrophages adhere.

17. After 1hr, aspirate off media into a 15 ml conical tube to collect non-adherent cells. Wash the adherent cells twice with **warm** HBSS (gently add the media, swirl the culture dish for 4-5 times and remove the media.

    Note: Non-adherent cells include mainly lymphocytes and neutrophils. Centrifuge the non-adherent cells suspension in 15 ml conical tube at 500xg in a refrigerated centrifuge set at 4 ^{0}C for 10 minutes. Resuspend the pellet in 1 ml RPMI media and transfer the cells to a labeled 2mL microcentrifuge tube. Centrifuge for 10 min at 500g. Discard the supernatant and freeze the cell pellet by snap freezing in liquid nitrogen. Store it at -80C locally.

18. Add 5 ml of cell detachment buffer to the dish and incubate for 3 min at 37^{o}C.

    Note: Preparation of cell detachment buffer:
    i) Solution A- Prepare 10mM EDTA in PBS (dilute the 0.5 mM stock of EDTA (sigma) with PBS)
    ii) Solution B - RPMI 1640 medium without antibiotics and serum
    iii) 1:1 mixture of Solution A and Solution B.
    iv) Aliquot the working solution in 5 mL volume aliquots and store at -20C.

19. Gently scrape adherent cells using a rubber cell scraper. Transfer cells to a sterile 15 ml Falcon tube.

20. Centrifuge the sample at 500xg in a refrigerated centrifuge set at 4 degrees for 10 min.

21. Decant supernatant, gently tap the tube and resuspend cell pellet in 3 ml of RPMI media **without** FBS. Count cells as described above.

22. Divide the cell suspension into two portions as described below.

    Portion 1: Aliquote a volume containing 3 million cells into a labeled 2 ml microcentrifuge tube. Centrifuge the sample at 500g in a refrigerated centrifuge set at 4^{0}C for 8 minutes. Immediately, remove the supernatant as much as possible and add the 0.6 ml of RLT buffer. Freeze at -80^{0}C Store for shipment to JHU for analysis. This is Aliquot 1 of the broncholalveolar lavage pellet.

    Portion 2: Rest of the cells will be used immediately for functional assays at JHU and SUNY.

    Note: Temple University: Because there are no functional assays conducted, transfer Portion 2 of the cell suspension into a 2 ml microcentrifuge tube (labeled as Aliquot 2 with a pre-printed label). Centrifuge the tube at 500g in a refrigerated centrifuge set at 4^{0}C for 8 minutes. Immediately, remove the supernatant as much as possible and snap freeze the sample in liquid nitrogen. Store at -80C. Ship to JHU.

23. **Procedure for cytospin slides:**

a. Dry the 6 cytospin slides in a desiccation chamber overnight. Use 3 cytospin slides for staining. Store the unstained slides after acetone fixation.
b. Staining Procedure: Use Diff-quick staining kit.

I. Solution A – Fixative: fix the slides into solution A for 15 seconds.
II. Solution B – Blue: Dip 5 times into Solution B
III. Solution C – Red: Dip 5 times into Solution C
IV. Wash in deionized H20 and air dry
V. Dip in xylene and cover slip

c. Unstained slides: Fix the cytospin slides by immering in cold high quality acetone for 10 min and air dry

d. Transfer the 3 stained and 2 unstained slides into the slide box and store it at -20^0C. Ship to JHU

e. Store 1 unstained slide locally at -20^0C

5.8.1 Protocol for bacterial clearance by alveolar macrophages

Supplies

- Clinical center will provide
  - Sterile glass beads
  - Polystyrene round bottom 12x75mm
  - 96 well plates
  - RPMI without FBS and without antibiotics
  - Pipette aid
  - Laminar flow hood
  - Pipettman

Procedure

Note: Bacterial growth conditions and growth curve must be established and optimized in each laboratory. Analysis of bacterial clearance can be performed in a 96-well plate.

1. Use portion 2 following macrophage purification and counting, aliquot $1x10^5$ cells in a total volume of 100ul of RPMI media **without** FBS and **without** antibiotics into wells of a 96-well plate. **At minimum, perform in triplicate.**
2. Add 50 CFUs of bacteria per cell (macrophage to bacteria ratio of 1:50; ~$5x10^6$ CFU per well) according to established growth curve of your laboratory.
3. Incubate for 4 h at 37 C.
4. Gently pipette the media several times and transfer to a sterile, labeled eppendorf tube.
5. For each well, perform a series of 6 serial dilutions, each 10-fold. (For dilutions of 1:10, 1:100, 1:1000, 1:10000, 1:100000, and 1:1000000).
6. Plate 100 ul of the 10^2, 10^3, 10^4, 10^5, and 10^6 serial dilutions on warmed LB plates. At least 2 plates / dilution.
7. Spread using sterilized glass beads, discarding beads after each use.
8. Incubate the plates at 37 degrees overnight.
9. Perform colony counts
10. Adjust counted colonies by dilution to determine CFUs of *Pseudomonas aeruginosa or H.influenza* in the sample.

5.8.2 Staining protocol for bacterial phagocytosis by flow cytometry:

Supplies

- Clinical center will provide
  - PBS, with EDTA 500ml
  - PBS with 1 % paraformal dehyde
  - RPMI without Phenol red
  - CO_2 incubator
  - CO_2 cylinder
  - Centrifuge
  - Access to Flow Cytometer
  - Rubber cell scrapper

Procedure

PERFORM ALL STEPS IN THE DARK IF POSSIBLE.

1. Following macrophage purification and counting, aliquot $2x10^5$ cells per well in a 24-well plate in a total volume of 300 ul of RPMI media **without** FBS and **without** antibiotics. Plate each sample **in triplicate**.

2. Add $1x10^7$ labeled bacteria per well according to dilutions necessary and established when bacteria were initially labeled.

   Note: A large quantity of FITC labeled *Pseudomonas aeruginosa* may be made in advance and can be kept at -20 for up to one year. It is best to use labeled bacterial from the same batch as to avoid variability. JHU can provide the FITC-labeled *Pseudomonas aeruginosa*.

3. Incubate for 60 min at 37 degrees with 5% CO2 in a dark incubator.

4. Wash the cells 3-times by aspirating media and washing with warm PBS (be careful to not use too high of a vacuum as to avoid aspirating cells.

5. Add 200ul of cold PBS with EDTA and remove cells my gently scraping with a rubber cell scraper and transfer to a labeled 1.5ml eppendorf tube.

6. Wash the cells 2-times by centrifugation at 500xg for 5 min and resuspend them in 100ul PBS with 1% paraformaldehyde

7. Store the cell suspension immediately at 4°C in the dark.

8. Analyze the cells on the flow cytometer as soon as possible.

5.8.3 Flow cytometry analysis of MARCO surface expression

Supplies

- Clinical center will provide
  - PBS, 500ml
  - Polystyrene round bottom 12x75mm
  - Fetal Bovine Serum, Heat Inactivated
  - Sodium Azide
  - MARCO, Human, mAb PLK-1 (Pri. antibody)
  - Goat Anti-Mouse IgG (Sec. antibody)
  - Bovine Serum Albumin (BSA)
  - Centrifuge
  - Access to Flow Cytometer

Procedure

Note: Indirect labeling requires two incubation steps; the first with a primary antibody followed by a compatible secondary antibody. The secondary (and not the primary) antibodies have the fluorescent dye (FITC, PE, Cy5, etc.) conjugated. Anti-MARCO (PLK-1) antibody by Hycult and DyLight 594 conjugated secondary antibodies from Jackson Immunoresearch are recommended.

1. Following macrophage purification and counting, aliquot $1x10^5$ cells in a total volume of 100ul of ice cold PBS, 10%FBS, 1% sodium azide in a polystyrene round-bottom 12x75 mm Falcon tube (perform in triplicate).

2. Add 10 μg/ml of the primary antibody (MARCO, Human, mAb PLK-1). Dilutions, if necessary, should be made in 3% BSA/PBS.

3. Incubate for 60 min at room temperature in the dark.

4. Wash the cells 3-times by centrifugation at 500xg for 5 min

5. Re-suspend the cells in the fluorochrome-labeled secondary antibody (Goat Anti-Mouse IgG; 1ug/ml;). If needed, dilute the fluorochrome-labeled secondary antibody in 3% BSA/PBS to obtain optimal concentration of the antibody.

6. Incubate for 30 minutes at room temperature in the dark.

7. Wash the cells 2-times by centrifugation at 500xg for 5 min and resuspend them in PBS with 1% paraformaldehyde

8. Store the cell suspension immediately at 4°C in the dark.

9. Analyze the cells on the flow cytometer as soon as possible.

   Notes: It is NOT advisable to measure MARCO surface expression on the same set of macrophage samples for bacterial phagocytosis. FITC and PE fluorescence may spill into each channel, necessitating increased number of cells and additional single stained samples to set compensations.

5.8.4 Anti-Inflammation and Steroid responsiveness

Supplies

- Clinical center will provide
  - 96 well plates
  - Pasteur pipette; Glass
  - Dexamethasone
  - Recombinant human TNF-alpha
  - Lipopolysaccharide from E.Coli 055:B5
  - Pipette aid
  - Laminar flow hood
  - Pipettman
  - Centrifuge
  - -80^0 C freezer

Procedure

1) Use PBMC aliquot 3 for this assay. Plate macrophage cells from PBMCs (50,000/well) or alveolar macrophages from BAL fluid (minimum 10,000/well) on 96-well plate. (Template shown in next page)

2) After 2 h of incubation, treat the cells with or without dexamethasone (1μM, 100nM, 10nM, 1nM, 0.1nM) for 10 min.

3) Cells were stimulated with TNF-α (1 ng/ml) or LPS (100 ng/ml) for 24h.

4) Centrifuge the plate at 500g for 10 min. (make sure you have rotor that can accommodate 96-well plate)

5) While centrifugation, label a new 96 -plate exactly in the same format. Transfer the media from each well to new label plate. Transfer the media as much as possible without the touching the bottom of the plate.

6) Seal the plate with parafilm and store at -80C and ship to JHU.

Analysis:

7) Level of IL-8 and other cytokines will be analyzed by ELISA at JHU.

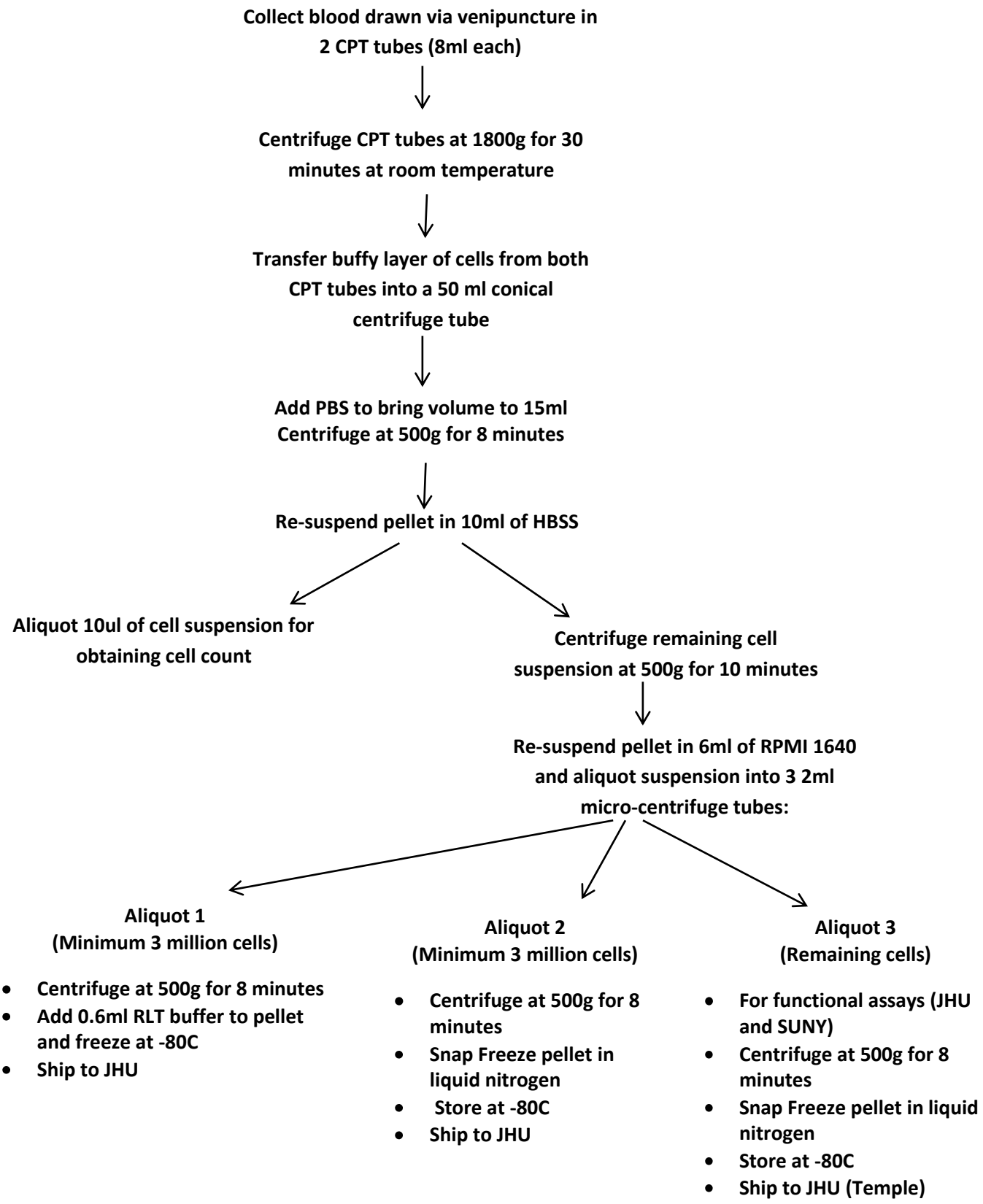
8) The IC50 value for dexamethasone (IC_{50}-Dex) for each sample will be calculated from sigmoid dose-response curves.

Note: Based on the number of cells available, the number of treatments may vary.
We don't anticipate any limitations with PBMC samples. However, alveolar macrophages may be limited.

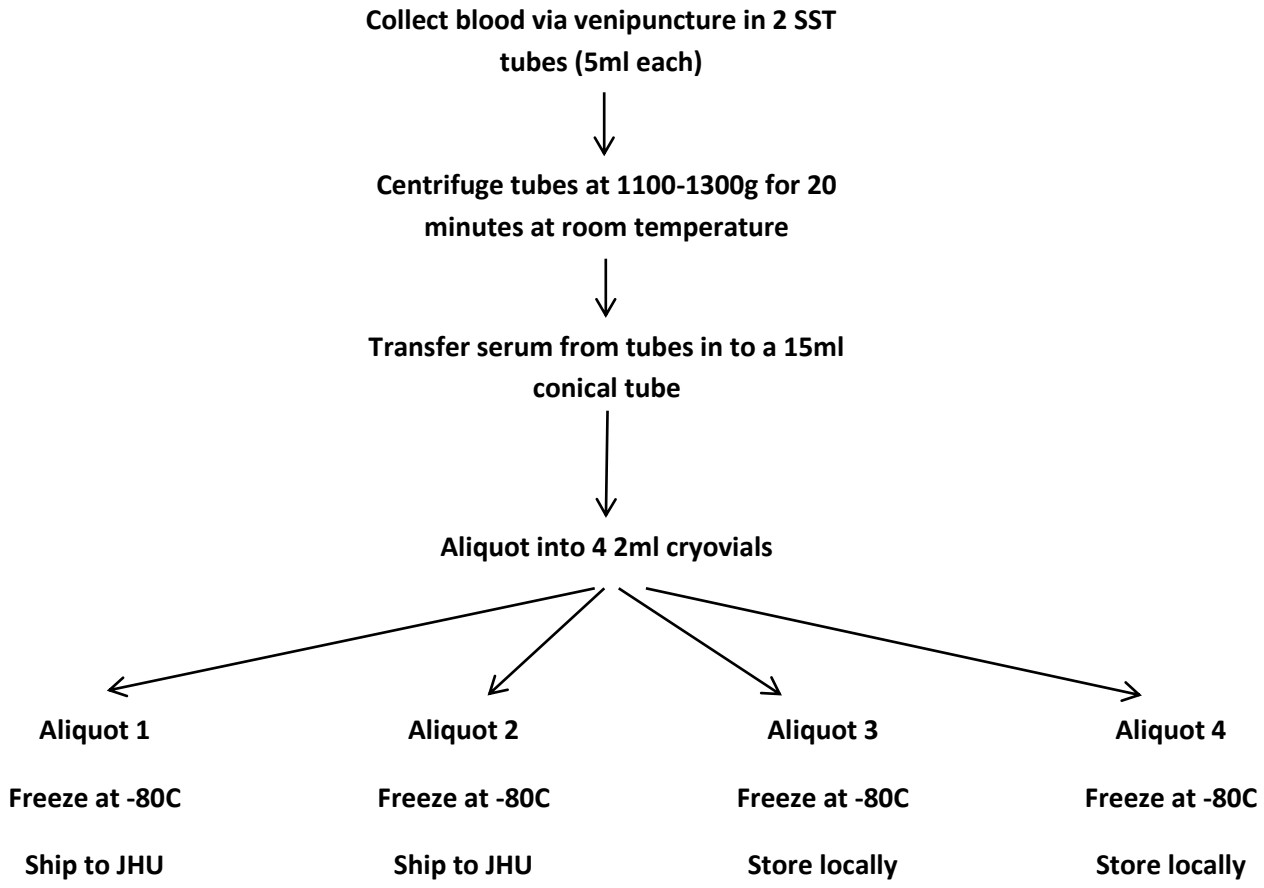
5.8.5 Template for plating PBMC and alveolar macrophages (Mac) for assessing inflammation and steroid responsiveness.

		1	2	3	4	5	6	7	8	9	10	11	12
PBMC	**A**	Veh	Veh	Veh	Dex (1nM)	Dex (1nM)	Dex (10nM)	Dex (10nM)	Dex (100nM)	Dex (100nM)	Dex (1000nM)	Dex (1000nM)	
	B	LPS (1ng/ml)	LPS (1ng/ml)	LPS (10ng/ml)	LPS (10ng/ml)	LPS (100ng/ml)	LPS (100ng/ml)	LPS (1000ng/ml)	LPS (1000ng/ml)				
	C	TNF (0.01ng/ml)	TNF (0.01ng/ml)	TNF (0.1ng/ml)	TNF (0.1ng/ml)	TNF (1.0 ng/ml)	TNF (1.0 ng/ml)	TNF (10 ng/ml)	TNF (10 ng/ml)				
	D	Dex + TNF-α	Dex + TNF-α	Dex + TNF-α	Dex+TNF-α	Dex + TNF-α	Dex + TNF-α	Dex + TNF-α	Dex + TNF-α				
	E	Dex + LPS	Dex + LPS	Dex + LPS	Dex+LPS	Dex + LPS	Dex + LPS	Dex + LPS	Dex + LPS				
Macrophages	**F**	Veh	Veh	Veh	Dex (1nM)	Dex (1nM)	Dex (10nM)	Dex (10nM)	Dex (100nM)	Dex (100nM)	Dex (1000nM)	Dex (1000nM)	
	G	LPS (1ng/ml)	LPS (1ng/ml)	LPS (10ng/ml)	LPS (10ng/ml)	LPS (100ng/ml)	LPS (100ng/ml)	LPS (1000ng/ml)	LPS (1000ng/ml)				
	H	Dex + LPS	Dex + LPS	Dex + LPS	Dex+LPS	Dex + LPS	Dex + LPS	Dex + LPS	Dex + LPS				

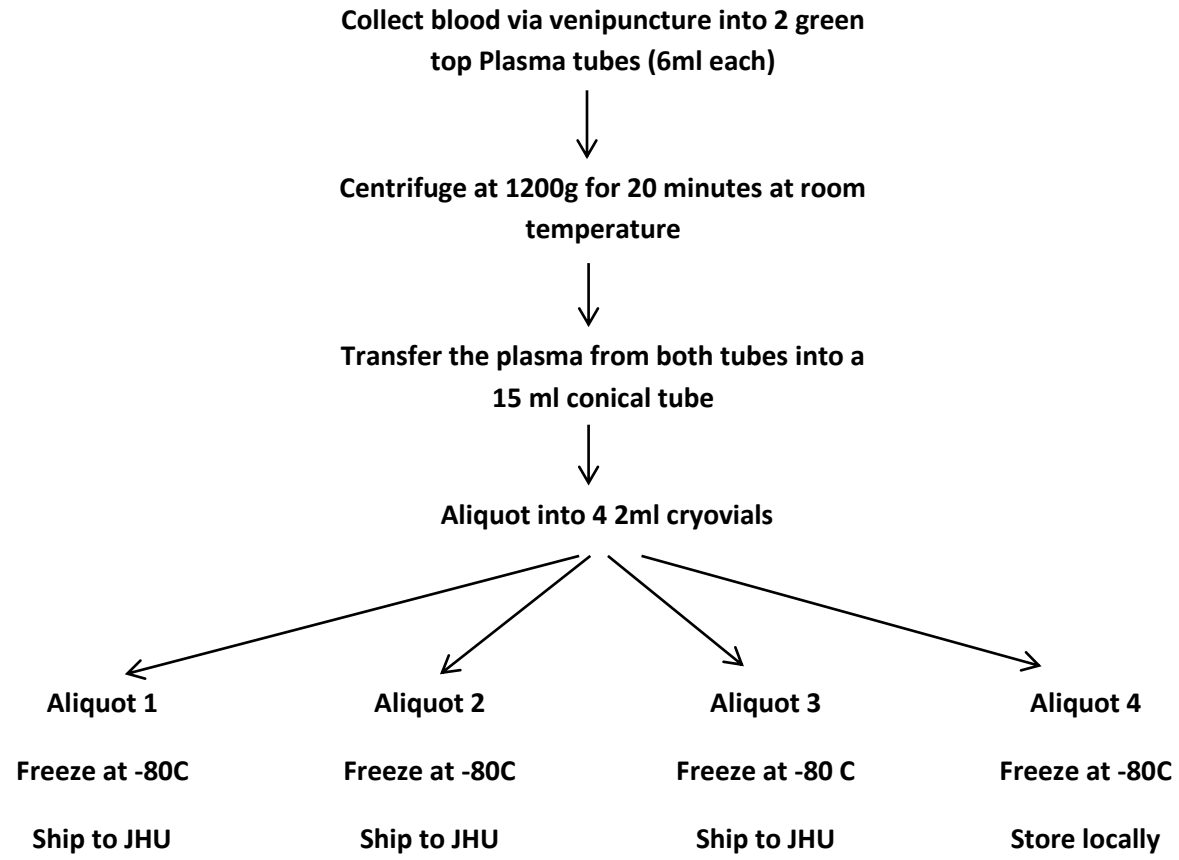
5.9.1 PBMC separation



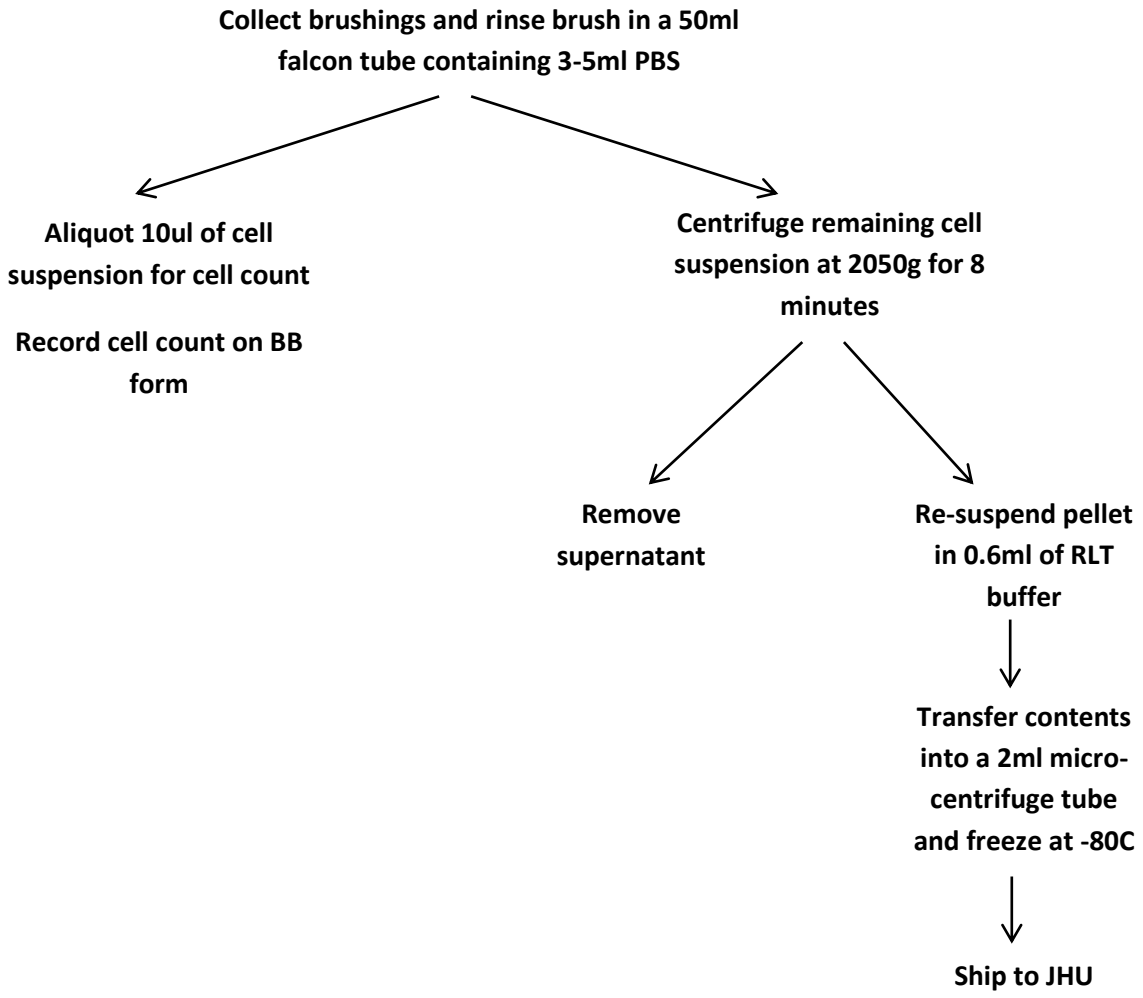
5.9.2 Serum separation



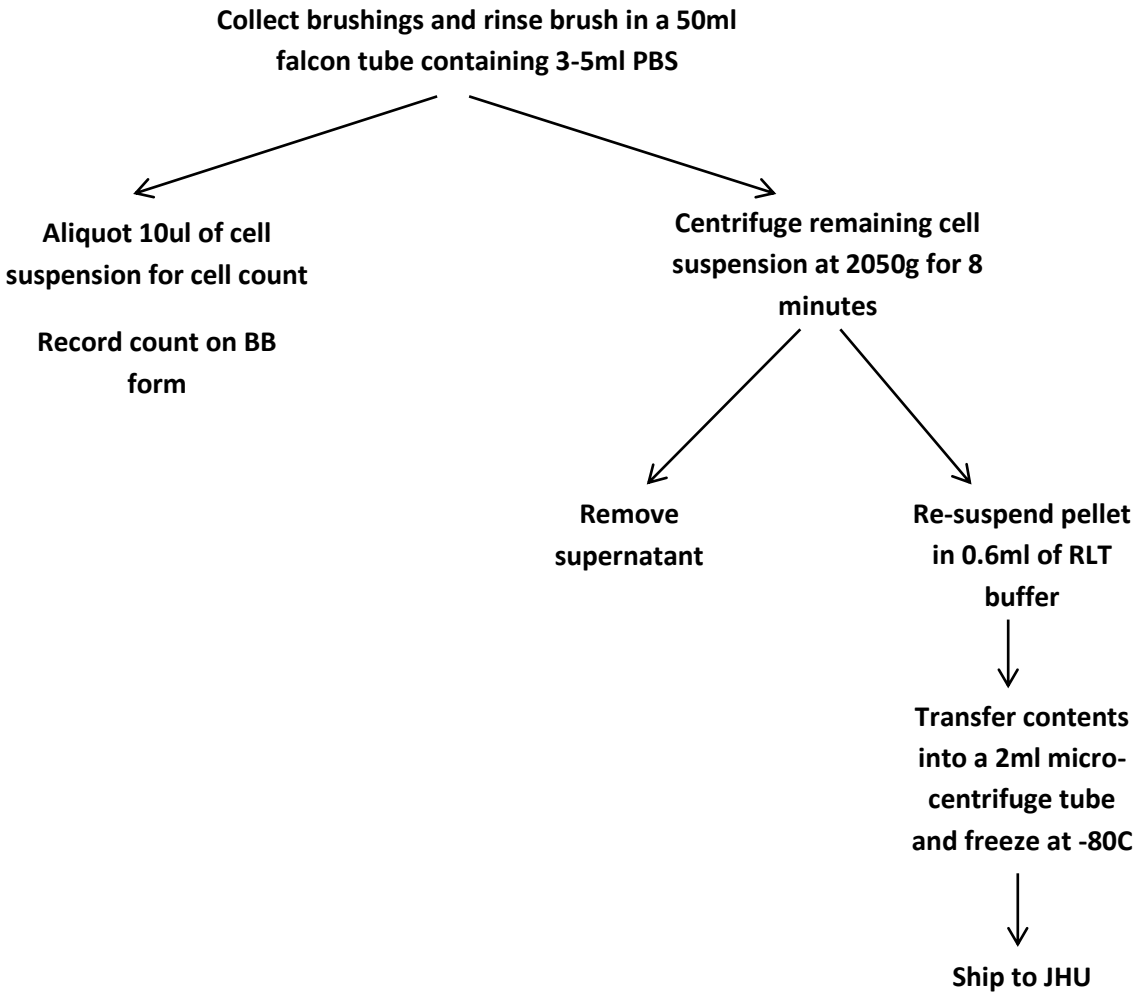
5.9.3 Plasma separation



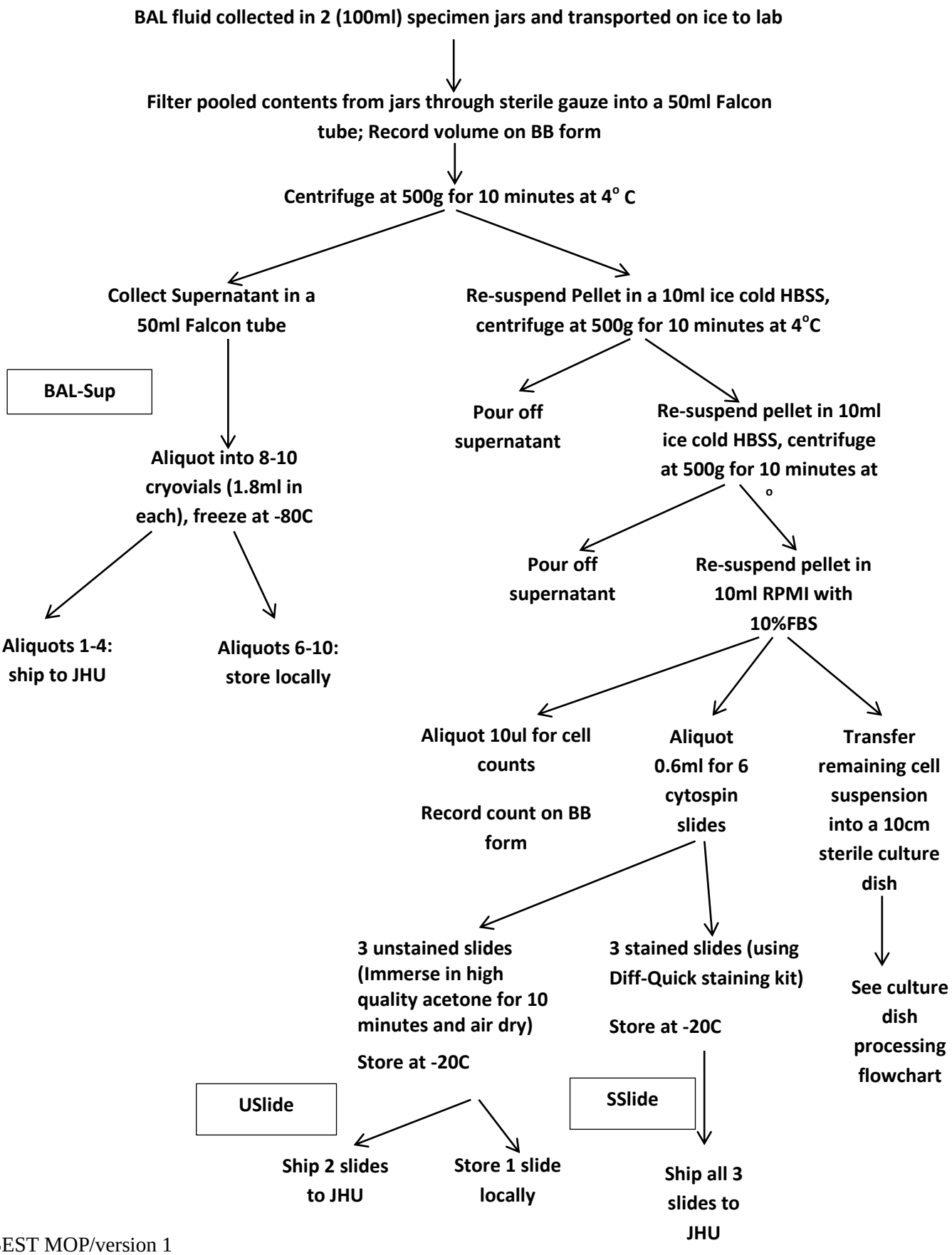
5.9.4 Nasal brushings (NB)



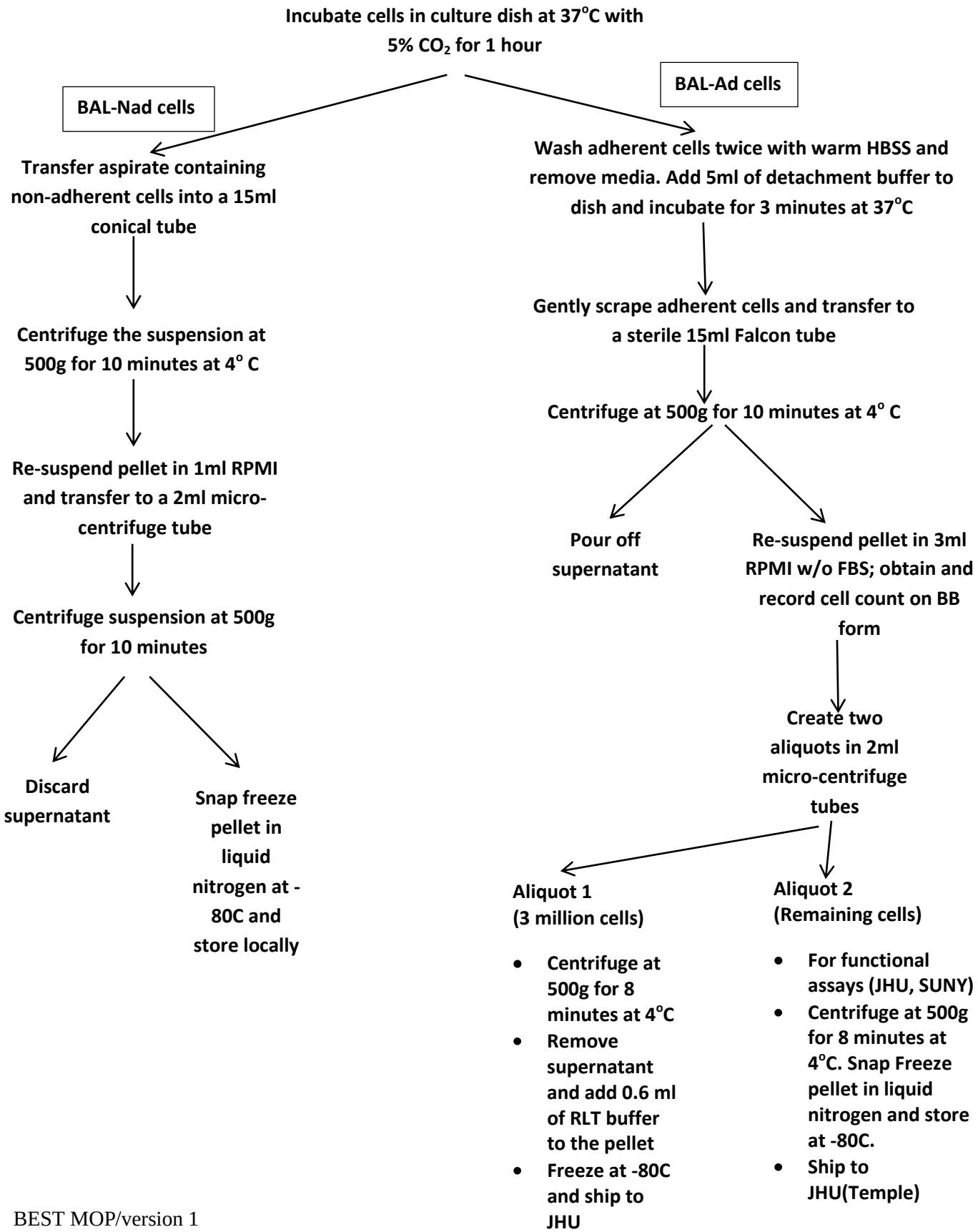
5.9.5 Bronchial brushings (BB)



5.9.6 BAL processing



5.9.7 Culture dish processing



5.10.1 Shipping overview

Cryovials and microcentrifuge tubes

- Specimen box
  - All specimens in cryovials or microcentrifuge tubes from an individual participant are to be shipped in a single box. Specimens should be placed in the box as specified in Section 5.10.2 (Master grid for shipping cryovials and microcentrifuge tubes)
  - If a specimen is missing leave the assigned space in the box empty

- Shipping
  - Once the participant has completed the trial and all existing specimens have been placed in the box, the box can be shipped to the analysis lab at JHU.

Slides

- Slide boxes
  - All 3 stained slides and 2 of the 3 unstained slides produced at a single visit should be placed in a slide box. The box should be labeled with a preprinted label containing the participant ID and visit number

- Shipping
  - Once the participant has completed the trial send the slide boxes for V3 and V5 to the analysis lab at JHU
  - Slide boxes from a single participant can be shipped along with the specimen box and 96 well plate (if applicable) from that participant in a single shipment

    or

    Slide boxes from a group of participants can be shipped together in a single shipment

96 well plates

- Plate preparation
  - Plates should be covered with Parafilm and frozen

- Shipping
  - Once the participant has completed the trial send plates for V3 and V5 to the JHU analysis laboratory

5.10.2 Master grid for shipping cryovials and microcentrifuge tubes

Box rows	*Box columns*									
		A	B	C	D	E	F	G	H	I
	BAL Sup	**BAL-Sup**	**BAL-Sup**	**BAL-Sup**	**BAL-Sup**	**BAL-Sup**	**BAL-Sup**	**BAL-Sup**	**BAL-Sup**	
	Visit ID	V3	V3	V3	V3	V5	V5	V5	V5	
	Aliquot #	1	2	3	4	1	2	3	4	
	BAL Ad. cells	**BAL- Ad. cells**	**BAL- Ad. cells**	**BAL- Ad. cells**	**BAL- Ad. cells**					
	Visit ID	V3	V3	V5	V5					
	Aliquot#	1	2	1	2					
	Bronchial Brushing(BB)	**BB**	**BB**							
	Visit ID	V3	V5							
	Aliquot #	1	1							
	Nasal Brushing(NB)	**NB**	**NB**							
	Visit ID	V3	V5							
	Aliquot #	1	1							
	EBC	**EBC**	**EBC**	**EBC**	**EBC**	**EBC**	**EBC**			
	Visit ID	V2	V2	V2	V4	V4	V4			
	Aliquot #	1	2	3	1	2	3			
	PBMCs	**PBMCs**	**PBMCs**	**PBMCs**	**PBMCs**	**PBMCs**	**PBMCs**			
	Visit ID	V2	V2	V2	V4	V4	V4			
	Aliquot #	1	2	3	1	2	3			
	Serum	**Serum**	**Serum**	**Serum**	**Serum**					
	Visit ID	V2	V2	V4	V4					
	Aliquot #	1	2	1	2					
	Plasma	**Plasma**	**Plasma**	**Plasma**	**Plasma**	**Plasma**	**Plasma**			
	Visit ID	V2	V2	V2	V4	V4	V4			
	Aliquot #	1	2	3	1	2	3			
	Other									
	Visit ID									
	Aliquot #									

Front of box

Key Points

- One participant per box
- If specimen is missing leave cell empty
- Don't send box to central lab until all specimens collected for the participant

5.10.3 Shipping cryovials and microcentrifuge tubes

Overview

- All existing cryovials and microcentrifuge tubes from a single participant should be placed in a cardboard specimen storage box. Once the participant has completed the trial the box is to be sent to the analysis lab at JHU

Shipping

- Government regulations require that personnel have job-specific training before offering a Dangerous Goods (including dry ice) shipment to FedEx or another air carrier. Personnel should receive training from their institution or make outside arrangements for a training session
- These instructions for shipping follow guidelines for Category B, Infectious Substances
- **DO NOT ship on Friday, Saturday, or day before a national holiday**

Shipping Supplies

- **Supplied by DCC**
  - Cardboard specimen storage box with partition
  - Styrofoam box with outer cardboard box
  - Plastic specimen bag (8" x 8")
  - Absorbent sheet
  - UN3373 label
  - Dry ice label (UN1845)
- **Supplied by clinic**
  - Dry ice
  - FedEx air bill

Shipping tasks

- Specimens must remain frozen throughout the packaging and shipping process
- All specimens in cryovials or microcentrifuge tubes from an individual participant are to be stored and shipped in a single box. Organize specimens in the specimen box according to the storage grid (section 5.10.2)
- If a specimen is missing leave the assigned space in the box empty
- Complete a Specimen Box Transmittal Sheet (ST) form for each box
- Place a specimen box and an absorbent sheet in plastic specimen bag
- Seal bag, insert copy of completed ST form in pocket and place bag in Styrofoam cooler
- More than one box and corresponding ST form can be included in a shipment
- Add a generous amount (at least 3 pounds) of dry ice and fill remaining space with padding (bubble wrap is acceptable)
- Affix proper labels on outside of box
  - UN3373 label
  - Dry ice label (with weight of dry ice and shipper/consignee name and address)

- Fed Ex air bill
  - Shipping address:
    - Joseph Vargas
      Johns Hopkins University
      Hygiene Building, Room E7410
      Johns Hopkins University
      615 N Wolfe Street
      Baltimore, MD 21205
      410-502-5633

  - Use Fed Ex account number: 2841-8497-1
  - Ship Priority Overnight
  - For item #6, Special Handling, check Dry Ice and fill in weight of dry ice in kilograms (3 pounds = 1.5 kg)
  - For item #6, Special Handling, check Yes, Shipper's Declaration not required
- Immediately upon shipment, notify lab by sending email to:
  rthimmul@jhsph.edu and dkencheg@jhsph.edu

Form (abbreviation)

- Specimen Box Transmittal Sheet (ST)

5.10.4 Shipping Slides

Overview

- 5 slides from a visit for a single participant should be placed in a plastic slide mailer. Once a participant has completed the trial all the slide mailers for that participant should be sent to the analysis lab at JHU

Shipping

- Government regulations require that personnel have job-specific training before offering a Dangerous Goods (including dry ice) shipment to FedEx or another air carrier. Personnel should receive training from their institution or make outside arrangements for a training session
- These instructions for shipping follow guidelines for Category B, Infectious Substances
- **DO NOT ship on Friday, Saturday, or day before a national holiday**

Shipping Supplies

- **Supplied by DCC**
  - Slide mailer
  - Styrofoam box with outer cardboard box
  - Plastic specimen bag (8" x 8")
  - Absorbent sheet
  - UN3373 label
  - Dry ice label (UN1845)
- **Supplied by clinic**
  - Dry ice
  - FedEx air bill

Shipping tasks

- Slides must remain frozen throughout the packaging and shipping process
- 5 slides (3 stained and 2 unstained) prepared m xxx obtained at a single visit from an individual participant are to be stored and shipped in a single box.
- Once a participant has completed the trial, ship all the slide mailers for that particular participant together.
- Complete a Specimen Shipping Sheet (SS) form for each shipment (a shipment can contain slide mailers for more than one participant)
- Place the slide mailers and an absorbent sheet in plastic specimen bag. Seal bag
- Insert copy of completed SS form in Styrofoam cooler
- Add a generous amount (at least 3 pounds) of dry ice and fill remaining space with padding (bubble wrap is acceptable)
- Affix proper labels on outside of box
  - UN3373 label
  - Dry ice label (with weight of dry ice and shipper/consignee name and address)

- Fed Ex air bill
  - Shipping address:
    - Joseph Vargas
      Johns Hopkins University
      Hygiene Building, Room E7410
      Johns Hopkins University
      615 N Wolfe Street
      Baltimore, MD 21205
      410-502-5633

  - Use Fed Ex account number: 2841-8497-1
  - Ship Priority Overnight
  - For item #6, Special Handling, check Dry Ice and fill in weight of dry ice in kilograms (3 pounds = 1.5 kg)
  - For item #6, Special Handling, check Yes, Shipper's Declaration not required
- Immediately upon shipment, notify lab by sending email to:
  rthimmul@jhsph.edu and dkencheg@jhsph.edu

Form (abbreviation)

- Specimen Shipping Sheet (SS)

5.10.5 Shipping 96-well plates

Overview

- At V3 and V5 BAL Aliquot 3 from PBMC collection is used for an ancillary functional assay to measure anti-inflammation and steroid responsiveness (JHU and SUNY). A 96-well plate is prepared and sent frozen to JHU for analysis

Shipping

- Government regulations require that personnel have job-specific training before offering a Dangerous Goods (including dry ice) shipment to FedEx or another air carrier. Personnel should receive training from their institution or make outside arrangements for a training session
- These instructions for shipping follow guidelines for Category B, Infectious Substances
- **DO NOT ship on Friday, Saturday, or day before a national holiday**

Shipping supplies

- **Supplied by DCC**
  - Styrofoam box with outer cardboard box
  - Plastic specimen bag (8" x 8")
  - Absorbent sheet
  - UN3373 label
  - Dry ice label (UN1845)

- **Supplied by clinic**
  - Dry ice
  - FedEx air bill

Shipping tasks

- 96-well plates must remain frozen throughout the packaging and shipping process
- A 96-well plate is prepared after Visits 3 and 5 for each participant at JHU and SUNY
- Once a participant has completed the trial, ship the 2 plates for that particular participant together in a single shipment.
- Complete a Specimen Shipping Sheet (SS) form for each shipment (a shipment can contain plates for more than one participant)
- Place the plates and an absorbent sheet in plastic specimen bag. Seal bag
- Insert copy of completed SS form in Styrofoam cooler
- Add a generous amount (at least 3 pounds) of dry ice and fill remaining space with padding (bubble wrap is acceptable)
- Affix proper labels on outside of box
  - UN3373 label
  - Dry ice label (with weight of dry ice and shipper/consignee name and address)

- Fed Ex air bill
  - Shipping address:
    - Joseph Vargas
      Johns Hopkins University
      Hygiene Building, Room E7410
      Johns Hopkins University
      615 N Wolfe Street
      Baltimore, MD 21205
      410-502-5633

  - Use Fed Ex account number: 2841-8497-1
  - Ship Priority Overnight
  - For item #6, Special Handling, check Dry Ice and fill in weight of dry ice in kilograms (3 pounds = 1.5 kg)
  - For item #6, Special Handling, check Yes, Shipper's Declaration not required
- Immediately upon shipment, notify lab by sending email to:
  rthimmul@jhsph.edu and dkencheg@jhsph.edu

Form (abbreviation)

- Specimen Shipping Sheet (SS)

5.11 Supplies list

No.	Consumables	Company	Catalog number
1	Pasteur pipette; Glass	Fisher	13-678-6B
2	Sterile gauze	BD Falcon	352350
3	Sterile glass beads (optional)	VWR	89001-052
4	Polystyrene round bottom 12x75mm (BD Falcon Cat#)	BD Falcon	
5	96 well plates	BD Falcon	353072
6	Rubber Cell Scraper	Sarsted	83.1830
7	15ml and 50ml conical centrifuge tubes	Any company	--
8	Sterile disposable pipettes (5, 10 and 25ml)	Any company	--

No.	Chemicals	Company	Catalog No.
1	PBS, 500ml	Invitrogen	10010-023
2	HBSS , 500ml	Invitrogen	14175-095
3	RPMI with phenol Red	Invitrogen	11875-093
4	RPMI without Phenol red	Invitrogen	11835-030
5	Tryphan blue	Media Tech. Inc.	25-900-CI
7	EDTA solution	Sigma	E7889
8	RLT buffer	Qiagen	79216
9	Formaldehyde, 16%, Methanol free	Polysciences, Inc.	18814
10	Fetal Bovine Serum, Heat Inactivated	Invitrogen	16140
11	Sodium Azide	Sigma	S8032
12	MARCO, Human, mAb PLK-1 (Pri. Antibody)	Hycult Biotechnology	HM2208
13	Goat Anti-Mouse IgG (Sec. antibody)	Jackson Immunoresearch	115-496-146
14	Dexamethasone	Sigma	D2915
15	Recombinant human TNF-alpha	R&D systems	210-TA-050
16	Lipopolysaccharide from E.Coli 055:B5	Sigma	L6529
17	ACK lysing buffer (for lysing RBCs)	Quality Biological, Inc.	118-156-721
18	Bovine Serum Albumin (BSA)	Sigma	A9647
19	Differential Quik Stain Kit	Any company	

No.	Instruments
1	Centrifuge with swing bucket rotor (Refrigerated and ambient temperature)
2	Centrifuge (for microcentrifuge tubes)
3	Pipette aid
4	Laminar flow hood
5	Hemocytometer
6	Pipettman
7	Multichannel Pipette (Optional)
8	-80^0 C and -20^0 C freezer
9	Ice machine
10	Small liquid nitrogen tank
11	Vortex
12	CO_2 incubator
13	CO_2 cylinder
14	Dessicator
15	Access to Flow Cytometer
16	Dry Ice
17	Fedex air bill

6. Study drug and adverse event reporting

6.1 Study drug
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6.2.1 Adverse event reporting..... 6-8
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6.1 Study drug

Procedures and timing

- Distribute assigned study drug blisterpack at V3
- Evaluate adherence to study drug assignment at P1, P2, and V4
- Account for blisterpack receipt, dispensation and return
- Document temporary or early termination of study drug
- Unmask participants to treatment assignment upon study completion or in unusual circumstances (i.e. emergency situation) prior to study completion
- Facilitate quality control of study drug by sending in Quality Control (QC) blisterpacks for analysis at quarterly intervals

6.1.1 Description of study drug and storage requirements

Treatment groups

- Sulforaphane, 25 micromoles
- Sulforaphane, 150 micromoles
- Placebo

Drug packaging and labeling

- 31 dose blisterpack
- 3 capsules (one dose) in each of the 31 blisters
- Blisterpack
  - Comes in heat sealed zip lock freezer bag with dessicant pack
  - Unique ID (B-1000 to B-1134)
  - Labeled with 2 study drug labels (examples below on next page); the second label has a tear-off portion to be affixed to the Blisterpack Dispensing and Capsule Counting Form (DD)

Drug storage requirements

- At clinic center/research pharmacy: -20^{o}C freezer (-70^{o}C is acceptable)
- At participant's home: in zip locked freezer bag in freezer section of refrigerator

Label

Broccoli Sprout Extracts Trial

Lot #: OFD5x10g Exp date: Mar 2013 **Blister pack ID: B-1000**

Contents: 31 doses (one dose is 3 capsules from one blister)
of broccoli sprout extract containing
25 micromol sulforaphane OR 150 micromol
sulforaphane OR of placebo

Dose: 3 capsules from one blister once daily by mouth

Store in -20^{o} C freezer in zip-locked plastic bag

Participant ID: ___ ___ ___ ___ ___

Participant Name: _____ Issue date: _____

Physician: _____ Emergency call: _____

Caution: New drug limited by Federal law to investigational use
Supplied by Johns Hopkins University, Baltimore, MD

Broccoli Sprout Extracts Trial
Study Medication

Blister pack ID: B-1000
Expiration date: March 2013
Lot #: OFD5 x10g

Detach and affix to DD Form
Broccoli Sprout Extracts Trial
Study Medication

Blister pack ID: B-1000
Expiration date: March 2013
Lot #: OFD5 x10g

6.1.2 Distribution of study drug to clinical centers

- Distributed by BEST central pharmacy at Johns Hopkins University Bayview Medical Center
- DCC will arrange initial blisterpack shipments
  - 30 study drug blisterpacks (sent in 3 staggered shipments of 10 blisterpacks)
  - 8 Quality Control (QC) blisterpacks (see section 6.7)
- Clinical center must record receipt of all blisterpacks (study drug and QC) on Blisterpack Accountability Log (BK)
- *If and when more study drug is needed, clinical center orders using online BEST Drug Distribution System accessible from the BEST Data System Main Menu*

6.1.3 Dispensing study drug and instructions to participants

Study drug dispensed after randomization at V3

- Obtain study drug assignment (blisterpack ID) from online randomization system
- Assigned blisterpack ID should correspond to a study drug blisterpack in stock at clinical center. *If assigned blisterpack is not at clinical center **STOP**; contact DCC immediately.*
- Complete Blisterpack Dispensing and Capsule Counting Form (DD); remove tear-off portion of blisterpack label and affix to DD form
- Complete entry on Blisterpack Accountability Log (BK) (record participant ID , name code and date of disposition)
- Fill in blisterpack label (participant ID, name, issue date, physician name, emergency phone number)
- Give participant blisterpack in zip lock bag packed in NFL insulated lunch bag with gel pack

In all but unusual circumstances participant will need only one blisterpack of study drug for the entire study. If a participant requires a second blisterpack to complete the study (e.g., original blisterpack was lost; second bronchoscopy (V5) cannot be completed within 31 days of randomization), contact the DCC for instructions.

Instructions to participants

- Store blisterpack in zip-locked freezer bag in refrigerator freezer
- Take one dose (3 capsules from one blister) every morning

6.1.4 Compliance monitoring and return of study drug blisterpacks

- Monitor study drug adherence at P1, P2, V4
  - At V4 also remind participant to
    - take study drug up until the day before V5 bronchoscopy
    - bring study drug blisterpack to V5 bronchoscopy
- At V5
  - Collect study drug blisterpack from participant
  - Photocopy blisterpack to document number of capsules remaining; attach photocopy to DD form
  - Complete DD form
- Returned blisterpacks should be stored until the end of the trial
  - Store in a secure locked location at ambient temperature
  - DCC will notify clinics when blisterpacks may be destroyed

6.1.5 Temporary withdrawal or early termination of study drug

- Possible reasons: adverse event, participant request
- Study drug temporarily stopped before participant completes study
  - Record information relating to temporary discontinuation of study drug on the Clinic Visit form (CV)
  - Complete an Unusual Event (UE) form or Serious Adverse Event Report (SR) form as appropriate
- Study drug is permanently stopped before participant completes the study
  - Collect study drug blisterpack from participant
  - Complete Blisterpack Dispensing and Capsule Counting Form (DD)
  - Complete an Unusual Event (UE) form or Serious Adverse Event Report (SR) form as appropriate
  - Continue to follow participant for all study visits (i.e., continue all relevant study procedures) through P3
  - If early unmasking is necessary refer to section 6.6 below

6.1.6 Unmasking

- 2 sealed Study Drug Assignment Envelopes for each blisterpack ID are provided by DCC
  - One to send to the participant after he/she completes the trial
  - One for the clinical center to use if emergency unmasking required
- Normal unmasking after participant completes the study (i.e., after P3)
  - Sealed Study Drug Assignment Envelope mailed to participant along with Participant Exit Letter
  - Study personnel remain masked

- Early unmasking (before participant completes study)
  - Possible reasons for early unmasking
    - An acute, severe reaction suspected to be related to study drug where knowledge of assignment will help to determine treatment
    - An overdose of study drug by participant or someone else
    - Request by physician or participant approved by BEST Study Leadership
  - Non-emergency unmasking procedures
    - Submit written request to the Director of the Data Coordinating Center, Janet Holbrook, PHD, MPH
      - Include the details of the situation and reason unmasking is desired
      - Fax request to 443-438-1377 or e-mail to bestdcc@jhsph.edu
    - Requests will be considered by study leadership
    - DCC will communicate the decision and, if appropriate, reveal the study drug assignment to the designated clinic personnel or the treating physician
    - Complete Serious Adverse Event Report (SR) form or Unusual Event (UE) form required as applicable to report the event and unmasking

  - Emergency unmasking procedures
    - As noted above, a set of sealed Study Drug Assignment Envelopes will be sent to clinic to use for emergency unmasking only
    - Sealed Study Drug Assignment Envelope may be given directly to treating physician

      OR
    - Clinic center staff (preferably not study personnel) may open Study Drug Assignment Envelope and communicate treatment assignment directly to treating physician
    - If possible, study personnel should remain masked
    - Complete Serious Adverse Event Report (SR) form or Unusual Event (UE) form required as applicable to report the event and unmasking

6.1.7 Quality Control

- 8 Quality Control (QC) blisterpacks distributed to each clinical center
  - Labeled with Clinic ID and numbered 1-8
  - Store with study drug blisterpacks at the clinical center/clinical center pharmacy
  - Record receipt on Blisterpack Accountability Log (BK)
- Per FDA requirement, at 3 month intervals Central Quality Control Lab will analyze capsules from a QC blisterpack
  - DCC will email request and shipping instructions to Lead Coordinator
  - Document shipment of QC blisterpack to Quality Control Lab on Blisterpack Accountability Log (BK)

Forms and logs (abbreviation)

- Blisterpack Accountability Log (BK)
- Blisterpack Dispensing and Capsule Counting Form (DD)
- Clinic Visit (CV) form
- Phone Contact 1 and 2 forms (P1 and P2)
- Unusual Event (UE) form *when applicable*
- Serious Adverse Event Report (SR) form *when applicable*

6.2.1 Adverse event reporting

Purpose

- Document symptoms and medical events participant experiences during the trial

By whom

- Coordinator and study physician

When

- P1, P2, V4,P3

Procedures

- Using Clinic Visit (CV) form or Phone Contact (P1 or P2 or P3) form as a guide, interview participant regarding symptoms or other medical events experienced during the time interval specified on form (Baseline symptoms are captured at V2)
- Additional reporting requirements for all events graded 'severe'
  - Severe grade events that meet the criteria of a Serious Adverse Event must be reported on a SR form as detailed in section 6.8.2
  - Severe grade events that do not meet the criteria of a Serious Adverse Event must be reported to the DCC on an Unusual Event (UE) form faxed to the DCC within 3 days of learning of the event

Serious v. Severe events: The terms "serious" and "severe" are not synonymous. The term "severe" is used to describe the intensity (severity) of an event; the event itself, however, may be of relatively minor medical significance (such as a severe headache). This is not the same as "serious", which is based on patient or event outcome or action criteria, usually associated with events that pose a threat to a patient's life or functioning. Seriousness (not severity) serves as a guide for defining regulatory reporting obligations. Severity and seriousness must be independently assessed.

Forms (abbreviation)

- Clinic Visit (CV)
- Phone Contacts
- Serious Adverse Event Report (SR)
- Serious Adverse Event Report Fax Coversheet (SF)
- Unusual Event (UE) form

6.2.2 Serious Adverse Event Reporting

Purpose

- Identification and expeditious reporting of serious adverse events

By whom

- Study physician is responsible for determining if an adverse event is a serious adverse event and whether the serious adverse event is related to study treatment
- Coordinator is responsible for timely submission of Serious Adverse Event Report (SR) form to DCC and providing updates or follow-up reports as necessary/requested

When

- Within 3 working days after study personnel become aware of the event an SR form must be faxed to DCC
- When appropriate, original SR form should be updated or new SR form completed as a follow-up report

Definition of Serious Adverse Event

A serious adverse event (SAE) is an adverse event that results in one of the following outcomes:

- Death
- Life threatening experience
- Inpatient hospitalization or prolongation of hospitalization
- Persistent or significant disability/incapacity
- Congenital anomaly/birth defect
- Important medical event that, based on appropriate medical judgment, may jeopardize the participant and may require medical or surgical intervention to prevent any of the outcomes previously listed in this definition

Procedures for reporting a serious adverse event

- Fill out SR form with available information, include a narrative description of the event, its clinical significance and any extenuating circumstances
- Fax SR form and Serious Adverse Event Report Fax Coversheet (SF) to DCC (fax number is on coversheet) within 3 working days of learning of the event
- DCC staff will confirm receipt of SR within 24 hours or the next working day. If receipt is not confirmed, contact the DCC.
- Key Serious Adverse Event Report (SR) into BEST data system within 5 working days
- The original SR form should be updated or a follow-up SR should be completed as needed
  - To provide additional information requested by the DCC/BEST Medical Safety Officer
  - When the serious adverse event is resolved, or if there has been a significant change in the participant's condition or the physician's judgment about the event since the previous report was filed. The study physician should use his/her judgment in deciding what is significant.
- If there is a study clinic visit after the event, record event on CV form at that study visit

Forms (abbreviation)

- Clinic Visit (CV)
- Phone Contacts
- Serious Adverse Event Report (SR)
- Serious Adverse Event Report Fax Coversheet (SF)

7. Data collection and forms completion

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7.1 Completing forms

7.1.1 General guidelines

- Use dark blue or black ink
- Written responses should be legible to other people
- Limit use of abbreviations
- All completed forms should be reviewed by clinic coordinator to ensure that
  - All items are answered
  - Written responses are legible
  - Data are consistent
- Never change the wording of questions, decimal places, or the unit for a response that is pre-coded on a form
- A response space should have only one letter or digit per space
- All forms must be signed off by a study physician or clinic coordinator who is certified for the study
- Numeric responses are to be right justified with all spaces completed. Use lead zeros as necessary. Example: 0 0 3 2
- Alpha or alphanumeric responses are to be left justified. If blank spaces remain, leave them blank. Example: P 1 2 __; D C C __
- Data on forms should always match database

7.1.2 Error correction

- Do not obliterate erroneous responses
- Never use White-out or erase a response
- Draw a single line through incorrect response and indicate correct response clearly, above or next to the erroneous response
- Use a different color ink; e.g., green or red, to make edits
- Staff member making changes to a form should initial and date change in the margin and provide a short explanation for the change; e.g., “error”, “participant changed mind”
- Update database with revised information

7.1.3 Rounding rules

- Responses should have only one letter or digit per space
- The number of spaces or location of a decimal point on a form are never to be added or changed
- If a response has a greater number of digits to the right of a decimal point than spaces allow, the response should be rounded as follows:
  - If the first digit following the last data space is less than 5, round down; e.g., if the form has spaces for a 2 digit response with one decimal between the digits (__.__), then 4.71 would be rounded to 4.7 and 4.14 would be rounded to 4.1
  - If the first digit following the last data space is 5 or more, then round up; e.g., if the response field is for 2 digits with a decimal between the digits (__.__), then 4.78 and 4.75 would be rounded to 4.8 and 4.15 would be rounded to 4.2

More examples:

- For a response field of three digits (__ __ __), 79.485 would be recorded as 079 and 79.584 would be recorded as 080
- For a response field of __ .__ _, 4.2745 would be recorded as 4.27 and 4.2754 would be recorded as 4.28

Note: Do not round responses unless the number of digits in a response field requires it.Otherwise, record data as collected.

If there are more whole integer digits than the response field allows, contact the Data Coordinating Center.

7.2 ID Codes

Codes

- Reference #
- Participant ID
- Namecode
- Visit
- Personnel Identification Number (PIN)
- Clinical Center ID

Reference #

- Assigned by data system after the second keying of a form is completed
- Unique to every form entered into the data system
- Recorded onto form by data entry personnel after form is entered into database

Participant ID

- 5 digit center alphanumeric code taken sequentially from the sheet of Clinic Labels
- Distributed to the clinical center by the Data Coordinating Center (see clinic label sheet - example)
- Unique # assigned to each potential participant who starts the Screening Visit (V1)
- All participant forms will be identified by that #

Namecode

- 5 character code, unique for every participant enrolled at a site
- Assigned by coordinator at participant registration
- Suggested assignment scheme
  - First letter of code is the first letter of the participant's first name
  - Second letter is the first letter of the participant's middle name
  - Third-fifth letters are the first 3 letters of the participant's last name
  - Use "X" to substitute for any missing letters
- Examples
  - John L. Doe = JLDOE
  - John Doe = JXDOE
  - Don Ho = DXHOX
- If two participants at a site have the same namecode, substitute an "X" for one of the letters, use last letter of the last name, or substitute a number
  - Jane W. Smith = JWSMI
  - Joseph W. Smithe = JXSMI or JWSME
  - John W. Smile = JWSMX or JWSM 2

Visit

- May be pre-printed on the form or label by the DCC or hand recorded
- Screening visit (first clinic visit) = V1; Randomization visit (third clinic visit) = V3, etc.
- N = not associated with a study visit; e.g., Serious Adverse Event Report

Personal Identification Number (PIN)

- Unique 3 digit alpha-numeric identification code for each clinic staff member completing data forms
- Assigned by DCC

- Staff members must be “registered” by being entered in the online directory for a PIN to be generated

Clinical Center ID

- 2-4 letter code identifying primary clinical center site

Clinic	Code
Johns Hopkins School of Medicine	JHU
University at Buffalo, The State University of New York	SUNY
Temple University	TU

7.3 Dataform audits and data quality queries

First randomization audits

- The full set of forms for V2 and V3 will be audited for the first patient randomization completed by a coordinator
- If problems are identified, forms from the second and possibly third randomization completed by the coordinator may be audited

On-going data audits

- Periodically during and after the clinical phase of the trial, the DCC will audit clinics
- DCC will send an email to a clinic requesting copies of forms for a particular participant(s)
- Within 2 weeks clinics should send copies of the requested forms (BEST Fed Ex number: 2841-8497-1)
- The DCC will review the data on the paper form and compare it to entries in the data system

Drug Accountability Audits

- Periodically, the DCC will request materials from centers to audit BEST study blisterpack supplies
- The DCC will email lead coordinators who are responsible for collecting the requested materials for their site and submitting to the DCC
- The DCC will request:
  - A copy of current Blisterpack Accountability (BK) logs
  - List of kit IDs of all kits currently at the clinic

Data Quality (DQQs)

- The DCC will generate a Data Quality Query (DQQ) for a discrepancy found during an audit. A separate DQQ is created for each discrepancy found in a form
- All DQQs will be posted on the DQQ Management System. To get to the DQQ page, go to the BEST website, www.besttrial.org and follow the link to the Data System page. Clinics will receive a message box, alerting them that new DQQs are posted
- Clinics should respond to the posted DQQs
- After reviewing the DQQs, investigate the problem (usually by inspecting the source document) and make any necessary changes to the data system, the paper form, or both
- Changes to the paper form must be made according to the error correction procedures outlined in Section 7.1.2
- Resolving DQQs
  - After necessary changes to paper forms and/or data system have been made, go to the DQQ page, click on "View/Edit DQQ", choose one of the available options to indicate how the DQQ has been resolved (i.e., data system edited, data form altered, etc) and click "Submit" button. The DQQ will be removed from the clinic's DQQ list
  - Contact DCC if there are questions concerning the proper resolution of a DQQ
- Many coordinators find it easiest to go to the DQQ page, print out their list of pending DQQs, investigate and resolve each one, and mark on the paper list how each DQQ has been resolved. Then, they go back to the DQQ system and mark each as resolved by choosing and submitting the appropriate response