***SJÖGREN'S INTERNATIONAL COLLABORATIVE CLINICAL ALLIANCE (SICCA)***

**APPENDIX A: LABORATORY SPECIMEN COLLECTION, PROCESSING AND STORAGE STANDARD OPERATING PROCEDURES FROM ORIGINAL CONTRACT**

## I. OVERVIEW OF SPECIMEN COLLECTION AND IDENTIFICATION

A variety of specimens will be collected from each patient enrolled in SICCA. Specimens will be collected by phlebotomy and during the oral and ocular examination. With the exception of the San Francisco Research Group all SICCA sites will be collecting the identical set of specimens (see Section D, page 11-4). Required laboratory tests are listed in Section C, Schedule of Laboratory Evaluations. Blood specimens are discussed in section II; section III contains information on processing and handling non-blood specimens, and section IV discusses supplies needed for processing, handling and storage of SICCA specimens.

### A. DESIGNATION OF LAB TESTS

Of the specimens collected, the majority of specimens will be processed immediately, stored, and batched shipped to UCSF. Where tests are performed will vary as well. Categorized in relation to timing and location, each laboratory test and its requisite specimens are designated as one of the following:

1. *Local Immediate* – process immediately through your institution's clinical laboratory (e.g., CBC and Diff).
2. *Central Save and Batch* – process and prepare for temporary local storage. The majority of specimens will be batched shipped to the SICCA repository at UCSF.
3. *Exam Site* – process and prepare immediately for testing in the clinic (eye exam for example).

### B. IDENTIFICATION OF SPECIMENS

1. Labels are to be used on each specimen.
2. The SICCA Specimen Bank at UCSF will pre-assign accession numbers to each SICCA site. A set of labels and specimen deposit forms will be sent to each site. Contact Yvonne De Souza at yvonne.desouza@ucsf.edu for additional labels and deposit forms.

3. Each site has been provided with a Bank deposit form and a set of labels for each patient. The labels are preprinted with an accession number, which begins with the prefix – **"FS".**

- Take one UCSF SICCA Specimen Bank deposit form and its matching set of labels. **Double-check to see if the accession numbers on the form match the accession numbers on the labels.**

- The specimen type is also printed on the labels. You must select the correct set of labels for each specimen type (see table on following page). For example, you are processing Whole Blood (coded as WB-EDTA), you will use the labels that have the code WB-EDTA and the corresponding Access#. **The Access# on the label must match the one on the Bank Deposit form.**

******Please note there is no label for the CBC vial (5 ml EDTA). This tube is to be sent immediately for testing at your site.**

| SICCA SPECIMEN BANK CODE | SPECIMEN TYPE |
|---|---|
| BX | Biopsy |
| PFR-L | Parotid Flow Rate – Left |
| PFR-R | Parotid Flow Rate – Right |
| RNA-L | RNA conjunctival imprint -left |
| RNA-R | RNA conjunctival imprint - right |
| Serum | Serum |
| Tears-L | Tears – Left |
| Tears-R | Tears- Right |
| WB-EDTA | Whole Blood, EDTA tube |
| WS | Whole saliva |

- Make sure you fill out the following information on each deposit form: SICCA Patient ID or PID, and date (s) specimen (s) collected. There are times when you may not be able to collect all the specimens at once; use the same deposit form and fill out the date when you have collected a specimen or specimens.

- Please note that there is a processing disposition column on the deposit form. This is a reminder of how many aliquots and the number of ml per vial you need to prepare for a particular specimen type. It also indicates how the specimen is to be stored.

- On the deposit form – for each specimen type please indicate the number of vials you have processed. For example, sera – you have **one** 15 ml conical and **six** .5ml cryovials, and **five** x 1 ml of sera. You have a total of **12 vials and a total of 15 ml of sera. For tears you will have one tube for left and 2 strips in the vial instead of # of ml**. For labial salivary gland biopsy you may have two vials with a total of 3 glands per vial**. You will record this as 2 vials and total of 6 glands instead of ml.**

- The person responsible for processing should initial each line.

- A **copy** of each UCSF SICCA Specimen Bank Deposit form must be made. Keep the original and send the corresponding copies with your specimen shipment to UCSF.

- **The bank disposition column is for the UCSF SICCA Specimen Bank only.**

- Label the tube by placing the adhesive side down on the **writing spot** of the cryovial or 15, 50 ml conical tubes. Place the Bank's Accession number down first and then wrap the label around the tube. This self laminates the label and it will not fall off in at ultra-low temperatures (-70 °C) or in liquid nitrogen (-190°C). Examples of labeled cryovials and conical tubes have been sent to each SICCA site for examination. See photo below.


## C. SCHEDULE OF LABORATORY EVALUATIONS FOR SS PATIENTS

***Note: Effective December 31, 2011 there will no follow-up visits.***

| TEST | BASELINE | TWO YEAR FOLLOW UP VISIT |
|---|---|---|
| **OCULAR** | | |
| Lissamine Green Staining | X | X |
| Fluorescein | X | X |
| Schirmer Strips | X | X |
| RNA Imprint (Denmark, Argentina, UK and UCSF only) | X | X |
| **ORAL** | | |
| LSG Biopsy – Focus Score | X | X |
| Unstimulated Saliva | X | X |
| Parotid Saliva | X | X |

| BLOOD TESTS | BASELINE | TWO YEAR FOLLOW UP VISIT |
|---|---|---|
| Anti –ANA | X | X |

| | | |
|---|---|---|
| Anti-SS-a | X | X |
| Anti-SS-b | X | X |
| IgG, IgM, IgA | X | X |
| Rheumatoid factor | X | X |
| HCV Ab | X | X |
| CBC - differentials | X | X |
| Complement, component C3, C4 | X | X |
| Serum – repository | X | X |
| Whole Blood – DNA Bank | X | None to be collected |
| Plasma and PBMCs (UCSF only) | X | X |

**SCHEDULE OF LABORATORY EVALUATIONS FOR BLOOD RELATIVES AND UNAFFECTED CONTROLS**

| TEST | BASELINE | TWO YEAR FOLLOW UP VISIT |
|---|---|---|
| **BLOOD** | | |
| Whole Blood or Oragene – DNA Bank | X | |

*Only new participants and recall participants (pSS patients and Level 2 controls) will have blood, saliva, biopsies, and tears collected. The blood relatives and unaffected controls will have only four, 10 ml purple tops collected for DNA.*

## D. SPECIMENS TO BE COLLECTED FOR SICCA REGISTRY- ALL SITES

| Tube Type | Number of Tubes and Tube Volume | Processing | Storage Conditions |
|---|---|---|---|
| SST (Come as gold, red, or tiger top colors) Clot activator and gel separator | 4 X 10 ml | 1 x 7ml (use 15 ml conical), .5ml x 6; remaining sera in 1 ml aliquots | -70°C |
| Lavender Has $K_3$EDTA (liquid) | 1 x 5 ml | Send to local lab for testing – CBCs with Diff | Not applicable, must be tested fresh |
| Lavender Has $K_3$EDTA (liquid | 4 x 10 ml | Aliquot into **four 15** ml conicals tubes. You will have four, 10 ml aliquots (some may have less than 10 ml) | -70°C |
| Tears-Right | 1 cryovial | Strip should be in | -70°C |

| | | | |
|---|---|---|---|
| | | half lengthwise and stored in cryovial. | |
| Tears – Left | 1 cryovial | Strip should be in half lengthwise and stored in cryovial. | -70°C |
| Whole Saliva | 1 x 50 ml conical | .5 ml x no. of ml collected | -70°C |
| PFR- R | 1 x 15 ml conical | .25ml aliquots | -70°C |
| PFR - L | 1 x 15 ml conical | .25ml aliquots | -70°C |
| Biopsy | | 1 –2 cryovials depending on number of glands- 3 glands/Nunc vial | Liquid nitrogen |

**ADDITIONAL SICCA SPECIMENS TO BE COLLECTED AT SPECIFIED SITES**

| **Tube Type** | **Number of Tubes and Tube Volume** | **Processing** | **Storage Conditions** |
|---|---|---|---|
| ACD (Yellow tops) (UCSF) Has Acid Citrate Dextrose | 4 X 8.5ml | Plasma – 1 ml aliquots PBMCs – 1 x $10^{6}$/ ml | Plasma -70°C PBMCs – liquid nitrogen |
| RNA Imprint (Argentina, Denmark, India, Johns Hopkins, Univ. of Penn., UK and UCSF) | 2 strips – left and right | One strip in cryovial containing RNA*later*® | -70°C |

## II. BLOOD SPECIMENS

## A. BLOOD DRAWING PROTOCOL

1. PURPOSE

- To ensure the most efficient use of the volume of blood that is available for participants enrolled in the study;
- To designate samples for the SICCA repository.

2. BACKGROUND

A study such as SICCA requires a standardized but flexible protocol for the priority of the use of limited samples. The order of blood draw has been standardized by national and international authorities because of the possibility of cross contamination between tubes due to different additives. Clinical laboratory quality improvement plans have significantly reduced analytic error so that “preanalytical" variability now represents the most important source of errors that can lead to inaccurate patient results.” Additionally, the National Committee for Clinical Laboratory Standards (NCCLS) specifies the following order for blood collection:

1. Plain serum tubes
2. Sodium citrate tubes for coagulation tests
3. Gel separator tubes (SST)
4. Heparin tubes / heparin gel separator tubes
5. EDTA tubes
6. Glucose preservation tubes.

There is an excellent paper to review on preanalytical variables and the web address for this paper is:

http://www.bd.com/vacutainer/pdfs/LabMed_jan2003_num01_vol34.pdf.

It is preferred that when aliquoting serum, whole blood, and saliva for shipment to the SICCA repository, vials are filled to the stated volumes. In the event that all aliquotted vials will not have the full volume, do not distribute the total volume evenly among all vials. Fill as many vials as possible to the stated volume. It is more important to have correct volumes in fewer vials than to have less volume in more vials.

3. MISSING AND REPEAT LAB TEST REQUIREMENTS

CBC and Diff are required and must be performed at each site’s local laboratory. If missing, sites are to make every effort to have the participant return within a two-week window for blood draw, or in the case of unreliable results, perform a redraw. These tests cannot be run on specimens that have been frozen. Other serology tests such as anti-nuclear antibodies, anti SS-A, and SS-B will be performed at Quest Diagnostics, San Jose, California.

## B. COLLECTION METHODS AND PROCDURES

### A. Preparation:

1. The participant should be made as comfortable as possible before commencing phlebotomy. Precise and quality technique is necessary to preserve the participants' veins and well-being. Phlebotomy should be performed using aseptic technique. Phlebotomy steps are as follows:

a. The phlebotomist should wash her/his hands and **wear gloves throughout the procedure.** It is strongly recommended that the phlebotomist wear a lab coat and mucous membrane protection. To prepare for venous blood collection and reduce participant burden, phlebotomists should assemble tubes in the order stated in Section C.

b. Identify yourself to the participant and positively identify the participant's SICCA ID.

c. Ensure the participant is comfortable and that their arm is supported. Ask the participant to make a fist by opening and closing their hand several times.

d. Apply the tourniquet to the participant's arm 2 inches above the site selected for venipuncture. Use only the tightness necessary for vein visualization; an excessively tight tourniquet will cause stasis of the blood and will give incorrect laboratory results or make the patient very uncomfortable.

e. If a satisfactory vein is identified in the antecubital area remove the tourniquet and proceed with aseptic technique. It is preferable to remove the tourniquet while preparing the alcohol, gauze, and tubes since the tourniquet will be on for such a long time.

f. Once the tubes, alcohol, and gauze have been assembled, reapply the tourniquet; disinfect the venipuncture site (and any of the phlebotomist's fingers that might come in contact with it). Working outwards in a circular motion wipe the site selected with alcohol and then wipe dry with gauze pad.

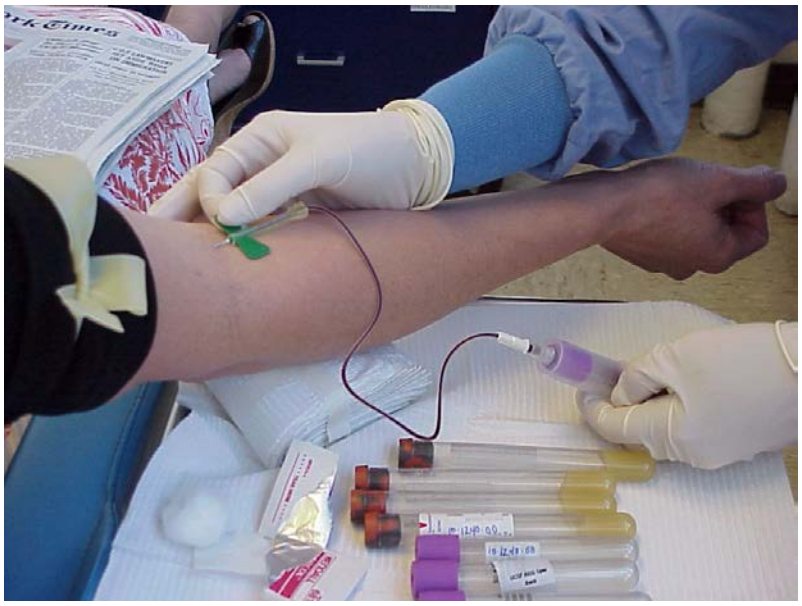
**B. Venipuncture:**

1. Inform the participant you are about to stick them with the needle and that he or she will feel a sting. At the same time, use firm pressure and insert the needle into the selected vein. **DO NOT JAB**. Fill tubes with blood in the order listed in Section C.

The tourniquet should be removed as soon as blood starts to flow – this avoids an increase in lipid values due to prolonged venous occlusion. If the blood flow slows or stops, try pulling the needle back slightly or advancing it in slightly. Make sure that you do not exit the vein –this will cause an unsightly and painful hematoma (bruising).

2. After all tubes have been filled, withdraw the needle carefully – avoid jabbing the participant. Under filled or over filled tubes can result in an incorrect blood/additive ration and thus impact clotting times. Apply sterile gauze to the venipuncture site. Ask the participant to apply firm pressure to the site.

3. Inspect the venipuncture site. If bleeding has stopped, apply a fresh piece of gauze and tape.



4. SICCA “Re-Stick” Policy: When a participant comes for a visit and the phlebotomist is unsuccessful in getting blood on the initial venipuncture, the tech will ask the participant for permission to attempt another site. If the second venipuncture is unsuccessful, the tech will ask the participant for permission to have a third attempt made by another tech. Technicians are limited to 2 sticks per participant. If the blood draw is unsuccessful and the participant does not grant permission for a repeat stick, a clinician must be notified before the participant leaves the clinic. Another appointment should be made for blood collection.

5. SICCA Participant Emergency Policy:

- If a participant states that she may faint before/during/after blood drawing:

    - Place the participant in a wheelchair and escort the participant to a stretcher holding area;
    - Place the participant in a bed with one side rail up and collect the blood specimen;
    - After blood is drawn, put up the side rail and check the participant's condition. The participant should be stable and well enough to move before allowing them to leave the clinic.
    - If the participant continues to feel faint while lying down (10 minutes after blood collection), check the participant's pulse and blood pressure. Contact a clinician to inform him/her of the participant's condition and location.

If a participant faints:

- Move the participant to a sitting position or a bed so she does not fall on the floor or otherwise injure herself;
- Notify a senior clinician so she can check the participant's pulse and blood pressure; senior clinician must monitor the participant and decide further action based on the participant's well being.

**C. Processing:**

1. Immediately after finishing venipuncture, label the tubes. The following information will need to be recorded on the label by the phlebotomist:

    - SICCA Study ID
    - Specimen Type (serum, whole blood.)
    - Date Specimen Collected
    - Study Visit Number - Indicate Baseline (B) or Follow-up visit (F)

2. Gently invert all tubes several times after they have been completely filled. Be sure to invert the 10ml (EDTA) Lavender tops **fifty times** to insure the blood and anticoagulant is thoroughly mixed.

3. Discard the needle and any other used supplies in appropriate containers. Clean the work area and prepare for the next participant.

4. Place the tiger-top SST and red-top tubes in a vertical position for 30 minutes to allow the clotting process to complete.

5. Fill out the UCSF SICCA Specimen Bank Deposit form. Make sure the SICCA ID number, Specimen Date, and Study Visit (record baseline or

follow-up) are included on the form. This form should be filled out by the clinician/phlebotomist.

6. Blood should be delivered to the lab for processing as soon as possible. Make a copy of the UCSF SICCA Specimen Bank Deposit form to accompany the specimens to the processing laboratory.

7. Blood must be processed within 4-6 hours of collection. If the Tiger-top SSTs cannot be processed within 6 hours of collection be sure to centrifuge the tubes at 1100 x g for 15 minutes in a horizontal rotor within six hours of collection.

## C. PHLEBOTOMY VOLUMES, ORDER OF BLOOD DRAW AND BLOOD SPECIMEN DESIGNATIONS

**Baseline Visits**

| Tube Color | Number of Tubes and Tube Volume | Test | Notes |
|---|---|---|---|
| Yellow ACD Tubes (UCSF Only) | 4 x 10ml | PBMCs and Plasma repository | |
| Tiger Top SSTs | 4 x 10ml | Anti ANA, Anti SSa and SSb, IgG, IgM, IgA, HCV Ab, C3, C4, Repository | Tests will be performed in San Francisco. |
| Lavender | 1 x 5ml | CBC with Diff | Mix thoroughly; test must be performed at **local lab** on freshly collected sample. |
| Lavender | 4 x 10ml | Repository and DNA extraction | Invert tubes 50 times to mix thoroughly. |

## D. BLOOD SPECIMEN DESIGNATIONS

Proper organization, packaging, shipping and handling of human blood borne pathogens
insure sample integrity while maintaining the timely and safe transfer of specimens. Specific packaging and shipping procedures must be followed in accordance with federal regulations (US) and International Air Transport Association (IATA) requirements. Always ship by overnight carrier. World

Courier has been designated as SICCA's shipper. World Courier will train each SICCA Research Group (Argentina, China, Denmark, Japan, and UK) in the proper shipping, labeling, and documentation for shipments to UCSF. IATA regulations require that the sender notifies the recipient of the dangerous goods prior to shipment. This is to alert the receiving party that the shipment is coming, and to ensure that prior arrangements have been made for someone to receive the shipment at delivery time. When contacting the recipient, include the courier company name, air bill number and the date of expected delivery. Refer to Section I of this chapter for standardized use of all specimen codes and volumes on all aliquots described below.

**1. Tests slated for local immediate processing are as follows:**

a. CBC with Diff & Platelets (2-5 ml EDTA plasma)

**2. Samples slated for Central Repository (UCSF SICCA Specimen Bank/Repository) Save & Batch:**

a. Plasma and Cell Repository (UCSF only)

b. Serum -A 10 ml tiger-top Serum Separator Tube (SST) draws only 8.3 ml of whole blood as the tiger-top SST contains a gel barrier (approx.1.5 ml). After centrifuging, the gel separates serum from the blood clot and prevents leakage of RBC and WBC contents into the serum. Phlebotomists should collect blood in more than one tiger-top SST since testing will require a full 10 ml of whole blood. Collect 4 X 10 ml tiger-top SSTs (with gel barrier). These will be aliquoted and stored at the central repository for future testing.

c. Whole Blood. Blood will be collected in four 10 ml EDTA (lavender) blood tubes for baseline visits only. The tubes must be thoroughly mix (**inverted 50 times** at least) and will be aliquoted as whole blood and shipped to UCSF for future DNA work.

**E. SPECIMEN TRACKING**

Labs at each SICCA site are to keep records of shipments and samples received from their clinics. A database should be created in each laboratory to track the identity and location of all SICCA specimens. Copies of shipping manifest should be made for record keeping. The following is a list of information that should be tracked in each laboratory:

- SICCA ID#
- Specimen type:
  - Serum
  - Whole Blood
  - Unstimulated Saliva
  - Parotid Saliva (L)
  - Parotid Saliva (R)

    - LSG Biopsy
    - Tears
    - RNA Imprints (Argentina, Denmark, United Kingdom and UCSF)
    - Plasma and PBMCs (UCSF only)
- Date and time of specimen collection
- Type of blood collection tube ( SST, EDTA-lavender.)
- Volume of liquid specimens
- Number of glands in each cryovial
- Location in freezers (freezer rack, box, row and column)

**F. PROCESSING SERUM FROM SST TUBES (TIGER TOPS)**

1. Blood should be collected aseptically in a tiger-top SST.

2. Gently invert the tube approximately five times immediately after collection to activate
the clotting process. Place the tube in a vertical position for 30 minutes to allow the clotting process to complete.

3. The tubes can be transported to the processing lab. Transport blood to the processing lab in non-breakable containers to prevent tube breakage.

4. Filled SSTs should be kept at room temperature and centrifuged within six hours of
collection. SSTs must be centrifuged at room temperature in a horizontal rotor (swing out head) at 1100 x g for 15 minutes.
5. Aliquot serum in 1 x 7ml (use a **15ml polypropylene conical tube**), .5 ml x 6 labeled cryovials, and the **remaining** sera should be aliquoted in 1ml aliquots. Label all specimens and ensure correct specimen codes and SICCA Specimen Bank ID number are on the labels. Verify the SICCA patient ID is on the UCSF SICCA Specimen Bank deposit form and the SICCA Specimen Bank ID number and specimen codes correspond to that patient.

6. Freeze at -70° C. Record location of specimens in freezer.

**G. PROCESSING WHOLE BLOOD FROM 10 ML LAVENDER TUBES (for future DNA work)**

1. Blood should be collected aseptically in a Lavender tube.

2. Gently invert tube approximately fifty times immediately after filling to make sure the
anticoagulant and blood are well mixed.

3. Processing labs should invert tubes five times immediately prior to aliquoting. Aliquot

whole blood into **10 ml aliquots in labeled 15 ml polypropylene conical tubes**. **Do not exceed 10 ml in each tube. Blood will expand when frozen**. Label all specimens according to protocol. The 50 ml tube to be used for aliquoting must be made of **Polypropylene** and is sterile. Suggested brands are at the end of this section.

4. Freeze at -70° C.

## H. PROCESSING OF PLASMA AND CELLS FROM ACD TUBES (UCSF Only)

1. Whole Blood, collected in ACD (Acid citrate dextrose) tubes. The maximum length of time for processing blood for **plasma storage** varies with the anticoagulant and the desired analyte: Acid Citrate Dextrose (ACD) plasma should be processed as soon as possible within 30 hours of collection. **PBMC retrieval** should be completed as soon as possible within 30 hours of specimen collection, again depending on the designated procedure and specifications in the protocol.

Plasma and PBMC separation and processing:

a. If both cells and plasma are to be retrieved, centrifuge tubes at 400xg for 10 minutes to separate cells and plasma. *(If only plasma is to be retrieved, centrifuge tubes at 800 to 1000xg for 10 minutes, then aliquot plasma per instructions.)*

b. Remove plasma carefully to avoid disturbing the cell layer. Transfer plasma to a sterile centrifuge tube. (Note: if multiple tubes of the same patient and same anticoagulant were drawn at the same time point, plasma should be pooled before storage aliquots are prepared.)

c. Centrifuge plasma again at 800xg for 10 minutes to remove any contaminating debris, cells and platelets.
d. Aliquot plasma into 1ml aliquots in sterile Starstedt cryovials.

e. Store plasma aliquots at -70°C.

f. Separate the PBMCs as described below. (The PBMCs can then be resuspended, counted and used for culture, stored as dry cell pellets, or stored as viable cell suspensions.)

Ficoll-Hypaque **Overlay** Method:

a.) If plasma was removed for storage, add a volume of sterile 1X PBS or HBSS (Hanks) equal to the volume of plasma removed. Mix

gently and thoroughly. This step will decrease clumping of the cells during separation.

b). Carefully and slowly pipet blood-HBSS or blood-PBS on top of ficoll-hypaque solution in sterile 15 or 50 ml centrifuge tubes. (Suggestion: gently allow mixture to flow down side of tube and pool on top of ficoll surface w/o breaking surface plane).

*c.)* N***ote****: The ratio of ficoll to whole blood may vary according to manufacturer's recommendations and laboratory experience. For example, some manufacturers recommend 4 parts diluted blood to 3 parts ficoll reagent, however practical experience in some labs has shown good results using 3 parts blood to 1 part ficoll.)*

d.) Centrifuge tubes at room temperature for 15 to 30 minutes at 300 to 800xg, in accordance with the density gradient solution manufacturer's recommendations. Note: The **centrifuge brake must be turned OFF** for the separation to be clean and to maximize retrieval of the PBMCs.

e.) Follow instructions under PBMC Washing.

Ficoll-Hypaque **Underlay** Method:

a.) If plasma was removed for storage, add a volume of sterile 1X PBS or HBSS equal to volume of plasma removed. Mix gently and thoroughly. This step will decrease clumping of the cells during separation.

b.) Carefully and slowly pipet ficoll-hypaque solution UNDER blood-HBSS or blood-PBS in sterile 15 or 50 ml centrifuge tubes.

***(Note****: The ratio of ficoll to whole blood may vary according to manufacturer's*

*recommendations and laboratory experience. For example, some manufacturers recommend 4 parts diluted blood to 3 parts ficoll reagent, however practical experience in some labs has shown good results using 3 parts blood to 1 part ficoll.)*

c.) Centrifuge tubes at room temperature for 15 to 30 minutes at 300 to 800xg, in accordance with the density gradient solution manufacturer's recommendations. ***Note****: The* ***centrifuge brake must be turned OFF*** *for the separation to be clean and to maximize retrieval of the PBMCs****.***

**PBMC WASHING**

a. After centrifugation, transfer the cloudy interface (PBMC layer) into appropriately labeled 50 ml sterile centrifuge tubes, by carefully "vacuuming up" the cells with a sterile plastic disposable pipette. Avoid aspirating the ficoll solution by maintaining the pipette tip just above the cell layer and SLOWLY drawing the cells up into the pipette.

b. Wash PBMCs by diluting the PBMC layer solution with at least an equal volume of 1XPBS or HBSS (Hanks). Centrifuge at 200-400xg for 10 minutes at room temperature.

c. Discard supernatant.

d. A second wash is optional, but is highly recommended.

e. Resuspend PBMC pellet in 1 to 4 ml of the appropriate complete medium or PBS, depending on size of pellet and the intended use. Cells should be counted immediately and prepared for cryopreservation. Place resuspended cells on ice when performing cell count.

**Determining Cell Concentration**

Cells counts are performed on the Coulter counter or by hand. Both techniques will be discussed. From a normal donor, one can recover 10-12 million mononuclear cells from one 10 ml ACD tube. Cell concentration: (count/ml) x (volume of resuspended cells) = Total Cells. One can safely use up to ten

million cells per 1 ml vial ($10^7$ cells). Do not use small volumes to preserve high cell concentration.

HAND COUNTS WITH A HEMACYTOMETER

a. Using aerosol resistant pipette tips, count and record the number of viable PBMCs per ml:

b. Pipette 20 ul of PBMC suspension into a 0.5 ml microcentrifuge tube. Add 80 ul of 0.4% Trypan Blue stain, making a 1:10 dilution. Mix carefully, avoiding aerosol creation.

c. Load hemacytometer and count the number of viable PBMCs in the four large corner squares. (See diagram on next page.) ***Viable PBMCs will be clear nonviable PBMCs will be blue.***

d. Calculate the number of PBMC/ml:

*($10^4$ = volume conversion factor to 1ml; $10^1$ = specimen dilution factor.)*

$$\frac{\text{PBMC in all four squares}}{4} \times 10^4 \times 10^1$$

example: $\frac{88 \times 10^5}{4} = 2.2 \times 10^6$ PBMC/ml

e. To determine the total number of cells, multiply the number obtained in (c) by the volume (mls).

f. automated counting may also be used. Follow manufacturer's instructions.

Examples:

- for 5ml suspension:

  $\frac{88}{4} = 22$

  $22 \times 0.5 \times 10^6 = 11 \times 10^6$ PBMCs, total.

- for 3ml suspension:

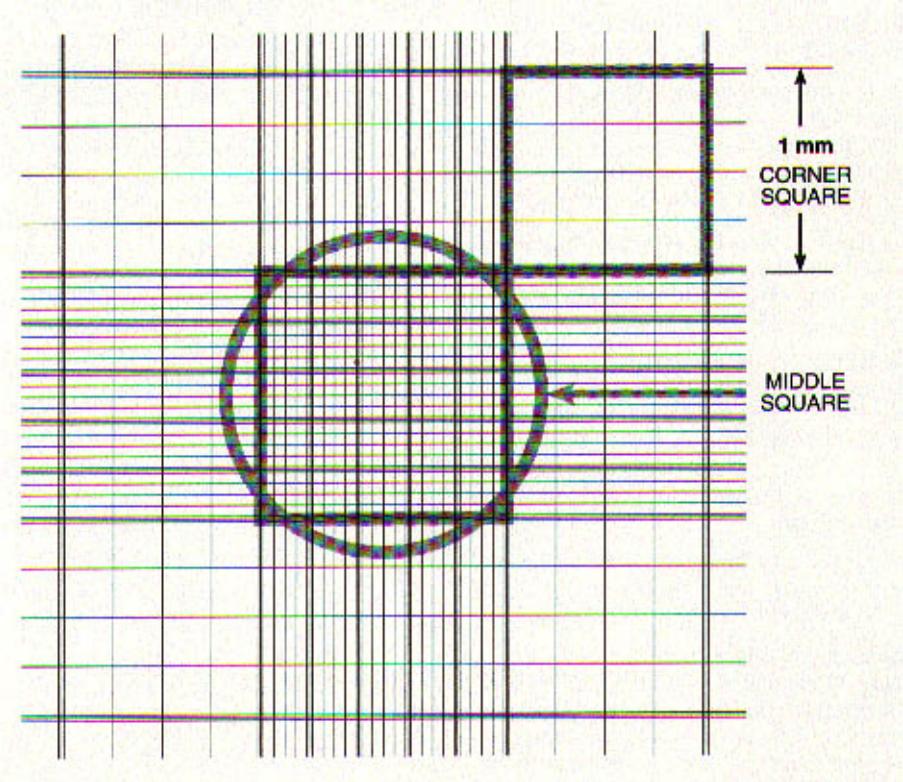
  $\frac{88}{4} = 22$

  $22 \times 0.3 \times 10^6 = 6.6 \times 10^6$ PBMCs, total.

- for 1ml suspension:

  $\frac{88}{4} = 22$

  $22 \times 0.1 \times 10^6 = 2.2 \times 10^6$ PBMCs, total.




**Count cells in the 4 corner 1mm squares. Include cells touching either the top line or left vertical perimeter line of any corner square. Do NOT count any cells that touch either the bottom line or right vertical perimeter line of any corner square.**

Calculate the number of PBMC/ml:

$10^4$ = volume conversion factor to 1ml
$10^1$ = specimen dilution factor

$$\text{PBMC/ml} = \frac{\text{PBMC in all four squares}}{4} \times 10^4 \times 10^1$$

example: $\frac{88}{4} \times 10^5 = 2.2 \times 10^6$

AUTOMATED CELL COUNTS – a Beckman/Coulter counter is used to count cells.

a. When you have your cell suspension in step 1.2.6, take 20 ul and place into a counting vial. Add 10ml of PBS and 3 drops of Manual Lysis media (Cat #5615). Follow manufacturer's instructions as to how to count cells.

Perform three readings and take the average cell count. Adjust cell concentration according to study protocol for cryopreservation.

**VIABLE PBMC CRYOPRESERVATION**

**NOTE: DMSO is a rapid penetrant thus the cryopreservation media, which contains DMSO must be applied ice cold and the cells must be on ice. If not, the DMSO will penetrate the cells at a rapid rate and the heat of reaction will rupture the cells.**

**Cryoprotective media = 90% Fetal Calf Serum (filtered and heat inactivated and 10% DMSO).**

a) Using aerosol resistant pipette tips, count cells and resuspend PBMCs to a concentration of $10x10^6$ PBMC/ml for SICCA with **cold** Cryoprotective Medium. Add the Cryoprotective Medium slowly, with constant gentle mixing, avoiding aerosol creation. The cells should be on ice when this step is performed to slow down the penetration of DMSO.

b) Dispense 1.0 ml aliquots (or less, depending on study's protocol) of the cell suspension into cryovials. Be sure cryovial caps are tightened.

c) Place the cryovials in a slow-freeze container (e.g., Mr.Frosty) in a -70°C freezer for 4-24 hours.

Transfer to vapor phase liquid nitrogen (-135°C) for long term storage. Alternatively, a controlled-rate LN2 freezing chamber (e.g. cryomed chamber) can be used**.** (When possible, avoid liquid phase storage due to safety concerns and to prevent possible problems with label adhesion failure.)

***NOTE:*** *To prepare and use the "Mr. Frosty":*

- *Remove the high-density polyethylene vial holder and foam insert from the polycarbonate unit.*
- *Add 250ml of 100% isopropyl alcohol to the fill line. DO NOT OVERFILL. (Avoid slopping the isopropyl alcohol on the labels, causes ink to run.)*
- ***Replace alcohol after every fifth use and document this reagent change.***
- *Carefully replace foam insert and vial holder.*
- *Place cryovials containing sample into holes in vial holder.*
- *Close "Mr. Frosty" and place in –70 freezer.*

## III. OTHER SPECIMEN TYPES (NON-BLOOD)

### A. PROCESSING OF WHOLE SALIVA

1. Unstimulated whole saliva will be collected in the clinic and the specimen must be kept **on ice** after collection. Whole saliva **must be processe**d **within one hour** of sample collection.

2. Whole saliva is placed into labeled cryovials as .5ml aliquots and stored at
–70°C.

### B. PROCESSING OF PAROTID SALIVA

1. Parotid saliva will be collected in the clinic and the specimen must be kept **on ice** after collection. Specimen must be processed the same day it was collected.

2. Do not mix the left and right parotid samples together. Indicate on the labeled cryovial which sample is from the right and the left parotid gland.

3. Parotid saliva is placed into labeled cryovials as 250µl aliquots. If there is less than 250 µl of saliva, be sure to **record** the amount on the SICCA Specimen Bank Deposit form.

### C. PROCESSING OF LSG BIOPSY

1. Paraffin Blocks

It will be an advantage to the research groups and the SICCA data and specimen banks if the following protocol can be followed at each SICCA site:

a. LSG specimens are sent to the local pathology laboratory where the tissue is accessioned under the patient's study number. The SICCA patient ID must be written on one of the slides of the paraffin block.

**Labeling** - Rub the paraffin wax off one of the sides of the block and pencil in the SICCA patient ID number. Write the letter "B" for baseline after the SICCA ID number or letter "F" for followup. The 4 unstained slides and H&E must be labeled with the SICCA patient ID and the letter "B" for baseline or letter "F" for follow-up.

On the following page you will see a photo of a paraffin block and H&E slides that is clearly labeled with a SICCA participant ID number.


b. That laboratory then processes the tissue, embeds it in paraffin, and prepares H&E-stained sections that are diagnosed by the local pathologist.

c. The **tissue block** is then loaned to the SICCA tissue bank (from which it can be retrieved at any time in the future should that be necessary), sent (by air mail) to the UCSF SICCA Specimen Bank along with **4 unstained slides and 2 H&E**. The SICCA Specimen Bank will be responsible for taking the blocks and slides to UCSF Oral Pathology where one slide will be stained for H&E staining and, if indicated, focus scoring.

The block should be wrapped in some type of protective wrapping and the 2 H&Es and 4 unstained slides should be sent in slide carriers (plastic or cardboard). The envelope used to send the entire shipment should be padded as well.

The block, 4 unstained slides, two H&E slide, and **SICCA Labial Salivary Gland (LSG) Shipment Form** (located on the administrative section of the SICCA website under “Manual of Procedures”, “Chapter 12”) are to be sent to the following address via registered airmail:

Ms. Danielle Drury
UCSF, Department of Orofacial Sciences
513 Parnassus Avenue, Box 0422
San Francisco, CA 94143-0422
USA

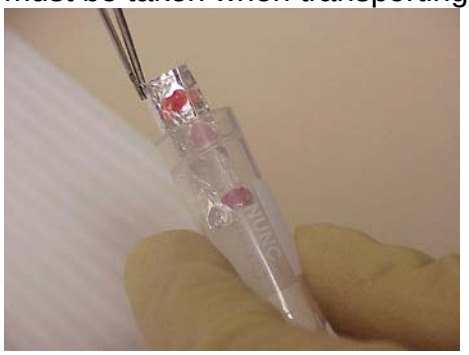
d. The supplemental report from this examination is then sent to the Research Group Director for incorporation in that patients study classification.

e. The paraffin tissue block will then be transferred to the UCSF SICCA Specimen bank for future sectioning to support research studies on paraffin-embedded tissue.

2. Frozen Labial Salivary Glands

a. The glands are prepared for freezing at the clinic to prevent the tissue from drying out.

b. Two to three glands are placed on an aluminum foil boat. The foil and glands are placed into a Nunc vial, capped, and immersed into liquid nitrogen (snap-freezing). Label the number of glands on the Nunc vial. Frozen labial salivary glands should be transported in a dewar that contains enough liquid nitrogen for an 8 hour day. Care must be taken when transporting the dewar to the laboratory.



c. Depending on the number of glands available for freezing, you may repeat step b. several times to freeze all of the glands collected.

d. All glands are stored in liquid nitrogen.

## D. OCULAR SPECIMENS

For more detail instructions on collection of ocular specimens, please go to Chapter 9 of this Manual of Operations.

1. Processing of Tears
   a. The tears are collected on Schirmer strips in the clinic.

   b. Each strip is cut in half, lengthwise. Each half is placed into a labeled cryovial and the label must indicate which eye the strip was taken from.

   c. Strips must be stored at –70°C.

2. Processing of RNA Imprints (Argentina, Denmark, United Kingdom and UCSF only)

Supplies Needed:

- RNA*later*® by Ambion (Cat # 7020 100ml 0r 7021 for 500 ml) for (Argentina, Denmark United Kingdom and UCSF)
- *MF- Millipore Filter mixed cellulose ester membranes, .45µm, 13 mm in diameter for Argentina, Denmark, United Kingdom and UCSF). Cat # HA P01300
- Cryovials – Starstedt 1.8 ml

a. Cut MF-Milliporemixed ceullose ester filter (white colored) in half.

b. After topical anesthesia with proparicaine and holding the eyelid open for twenty seconds to allow the ocular surface to dry, place one-half of the membrane (dull side down) against the temporal bulbar conjunctiva

c. Run the closed forceps tips against the surface of the membrane, then grasp the edge of the membrane and peel it off from the conjunctiva.

d. Place the membrane in a 1.8ml Sarstedt cryovial. The cryovial must be filled to near capacity with the RNAlater® reagent to ensure constant immersion and preservation of the collected samples.

e. Repeat the procedure for the nasal bulbar conjunctiva and place the membrane in the same tube containing RNAlater® reagent.

f. The cryovials should be placed on ice as soon as the specimens are collected and then transported to the laboratory where they are left at

4°C for 24 hours. The next day the cryovials are transferred to storage at –80°C until they are ready for shipment to UCSF.

g. Send the frozen imprints to UCSF with other SICCA specimens.

## IV. MASTER SUPPLY LISTS

The following is a list of supplies for the processing, handling, and storage of SICCA specimens. The vendors listed may not be available in some or all SICCA sites. SICCA Research Groups are not required to purchase from a particular vendor but rather to go to the referenced vendor's web site to see what the actual product looks like. Use your local vendors or hospital/university purchasing staff for assistance. If you have questions in regards to supplies, please email Danielle Drury (danielle.drury@ucsf.edu) or Yvonne De Souza (yvonne.desouza2ucsf.edu) for assistance. The standard operating procedures for the oral and ocular examinations also have supply lists.

| **ITEM** | **VENDOR (for reference only)** | **CATALOGUE NUMBER** |
|---|---|---|
| **Phlebotomy Supplies** | | |
| Yellow top vacutainers, 8.5ml, Acid Citrate Dextrose additives (ACD) for PBMCs and plasma | Fisher Scientific https://www1.fishersci.com/index.jsp<br><br>Vacutainer blood tubes are made by Becton Dickinson, go to their web site for blood tube information. http://www.bd.com/vacutainer/products/venous/tube_guide.asp | 0268426 |
| SST vacutainer, 10ml – (gold, red or tiger tubes) – Clot activator and gel for serum separation. If there are no SST tubes available, then purchase a tube (usually red colored stopper) that is specific for the collection of serum. | Fisher Scientific | 0268398 |
| Purple or lavendar top vacutainer, 5ml $K_3$EDTA (liquid) | Fisher Scientific | 026852C |
| Purple or lavender top vacutainer, 10ml, $K_3$EDTA (liquid) | Fisher Scientific | 0268384 |

| | | |
|---|---|---|
| Butterfly needle, 23 gauge | Fisher Scientific | 02664 |
| Vacutainer Holder | Fisher Scientific | 22289953 |
| Tourniquets | Fisher Scientific | 152352 |
| Cotton balls | Fisher Scientific | 07886 |
| Alcohol swabs | Fisher Scientific | 0666962 |
| Bandaids | Fisher Scientific | 19062477 |
| Gloves | Fisher Scientific | 1146267C |
| 5.1 cm x 5.1 cm cotton gauze | Local vendor | |
| **Specimen Processing Supplies** | | |
| Cryovial, Nunc (for LSG biopsy) | Fisher Scientific<br>https://www1.fishersci.com/index.jsp<br>or Nalgene Nunc Sci<br>www.nalgenunc.com | Fisher # 12565167N<br><br>Nunc # 377267 |
| 15 ml conical, polypropylene, there are many brands to use – Fisher, Corning, Sarstedt | Fisher Scientific<br>Corning<br>http:www.corning.com/lifesciences<br>Sarstedt<br>http://www.sarstedt.com | #0553859B<br>#43071<br><br>#62.548.004PP |
| 50 ml conicals, polypropylene<br>other brands to use include Sarstedt and Corning | Fisher Scientific<br>Corning<br>http:www.corning.com/lifesciences<br>Sarstedt http://sarstedt.com | #0553860<br>#430829<br>#62.554.001PP |
| 5ml aspirating pipette | Fisher Scientific | 1367522 |
| 5ml Serology pipette | Fisher Scientific | 1367522 |
| 10ml Serology pipette | Fisher Scientific | 1367520 |
| 25ml Serology pipette | Fisher Scientific | 13-668-2 |
| Histopaque for PBMCs | Sigma<br>www.sigma.sial.com | 10771 |
| Hanks BSS1x, w/o CA,MG, 500ml for PBMCs | Fisher Scientific | MT21021CV |
| Cryogenic vials, serum 2.0 ml, w/cap, w/writing space, w/graduations. For sera, tears, salvia, RNA imprints. | Sarstedt<br><br>http://www.sarstedt.com | 72.694.006 |
| Freezer box 5.1 cm x 5.1 cm | Fisher Scientific | 1167824A |
| Freezer box grid, 10 x 10 | Fisher or World Courier will supply these boxes and 10 x 10 grids | 1167824C (Fisher) |

| | | |
|---|---|---|
| Freezer box for 15 ml conical | Local vendor | |
| Grid for 15 ml conicals 6 x 6 | Local vendor | |
| Freezer box for 50 ml conicals - 148mm x 148 mmx 118 mm, includes 4 x4 grid insert | Sarstedt<br>Or local vendor | 95046916 (Sarstedt) |
| **Saliva Collection Supplies** | | |
| Carlson-Crittenton Cups | Stone Machine Co. | Contact UCSF |
| Citric acid | Local vendor | |
| Sterile water | Local vendor | |
| Tygon tubing (inner diameter is 1.6mm, outer diameter is 3.2mm and wall thickness is .8mm) 15 meters in length | VWR<br><br>http://vwrsp.com/catalog<br>or<br>http://www.vwr.com/index.htm | VWR Part # 63010-232<br>or<br>Tygon part number S-50-HL |
| Syringe cannula, blunt, plastic | Becton Dickinson<br>http:// www.bd.com | 303345 |
| Luer Lok 3 ml syringe | Becton Dickinson<br>http:// www.bd.com | 5585 |
| Rubber atomizer bulb with a flat base | VWR<br>http://vwrsp.com/catalog<br>or<br>http://www.vwr.com/index.htm | 56315-083 |
| Timer | Local vendor | |
| Ice container | Local vendor | |
| Topical anesthetic | Local vendor | |
| Local anesthetic(1-100,000 2%) | Local vendor | |
| Local anesthetic (1-50,000 2%) | Local vendor | |
| Syringe for anesthetic | Local vendor | |
| Disposable needle for anesthetic<br>30 gauge, monojet | Local vendor | |
| Scalpel #15 blade | Local vendor | |
| Adson tissue forceps(iris) | Local vendor | |
| Iris Scissors | Local vendor | |
| Needle holder | Local vendor | |
| Suture scissors | Local vendor | |
| Suture material 0000 plain gut or 4-0 Coated vicryl suture | Ethicon http://www.ethicon.com | Gut # 654-4199<br>Vicryl # 654- |

| | | |
|---|---|---|
| | | 2135 |
| 5.1 cm x 5.1 cm cotton gauze | Local vendor | |
| Binder Clips | Office Depot<br>http://www.officedepot.com/<br>Local Office supply Vendor | 308957 |
| **Ocular supplies** | | |
| | | |
| Schirmer tear strips | EagleVision<br>http:// www.eaglevis.com | 0039 |
| Lissamine Green | | Contact UCSF |
| Fluorescein | | Contact UCSF |
| RNA Imprint– mixed cellulose ester membrane, .45 micron, 13 millimeter diameter | Millipore<br>http://www.millipore.com | Cat # HA P01300 |
| Proparicaine | Local vendor | |
| RNAlater® | Ambion<br>http://www.ambion.com | Cat # 7020 100ml 0r 7021 for 500 ml |

## V. SICCA SPECIMEN COLLECTION & PROCESSING TABLE

| Specimen Type and Tube Type | Number of Tubes and Tube Volume | Processing | Storage Conditions |
|---|---|---|---|
| **SERUM** - SST (gold, red or tiger tubes) – Clot activator and gel for serum separation. If no SST tubes available, then purchase a tube that is specific for the collection of serum. | **4 X 10 ml** | 1 x 7ml (use 15 ml polypropylene conical), .5ml x 6; remaining sera in 1 ml aliquots | -70°C |
| **Whole Blood** Lavender (EDTA) $K_3$EDTA (liquid) | **1 x 5 ml** | Send to local lab for testing – CBCs with Diff | Not applicable, must be tested fresh |
| **Whole Blood** Lavender (EDTA) $K_3$EDTA (liquid) | **4 x 10 ml** | Aliquot into **four polypropylene 15** ml conicals tubes. You will have four, 10 ml aliquots (some may have less than 10 ml) | -70°C |
| **Tears – Right** | 1 cryovial | Strip should be in half lengthwise and stored in cryovial. | -70°C |
| **Tears – Left** | 1 cryovial | Strip should be in half lengthwise and stored in cryovial. | -70°C |
| **Whole Saliva** | 1 x 50 ml conical | .5 ml x no. of ml collected | -70°C |
| **PFR- R** | 1 x 15 ml conical | .25ml aliquots | -70°C |
| **PFR - L** | 1 x 15 ml conical | .25ml aliquots | -70°C |
| **RNA Imprint – R** (Argentina, Denmark, India, Johns Hopkins, Univ. of Penn., UK, and UCSF only) | 1 cryovial containing RNAlater® | Place on ice after collection, transport to lab and leave at 4ºC for 24 hours. The next day place the vial in a-70°C freezer. | -70°C |
| **RNA Imprint – L** (Argentina, Denmark, India, Johns Hopkins, Univ. of Penn., UK, and UCSF only) | 1 cryovial containing RNAlater® | Place on ice after collection, transport to lab and leave at 4ºC for 24 hours. The | -70°C |

| | | | |
|---|---|---|---|
| | | next day place the vial in a-70°C freezer | |
| **Biopsy-frozen** | | 1 –2 cryovials depending on number of glands- 3 glands/Nunc vial | Liquid nitrogen |
| **Biopsy-paraffin** | | The following is shipped to UCSF – one block, 2 H&E and 4 unstained slides. | Room temperature |
| **PBMCs and Plasma (UCSF only**) 4 yellow tops (Acid citrate dextrose) | | | Plasma - -70°C PBMCs – Liquid Nitrogen |

Only new participants and *recall participants (no more recall participants will be seen after December 31, 2011) will have blood, saliva, biopsies, and tears collected. The blood relatives and unrelated controls will have saliva collected using the Oragene kits or blood collected in four, 10 ml purple tops for DNA.

* Effective 11/19/2010 (see SICCA Communication Memo #47) collection of 4 x 10ml EDTA blood tubes for DNA will take place during the **baseline visit only**.