LABORATORY FOR ASSESSMENT OF MUCOSAL IMMUNE RESPONSES
INDUCED BY AIDS VACCINES IN CLINICAL TRIAL VOLUNTEERS

Collection and Processing of Mucosal Specimens

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This copy of the Manual for Collection and Processing of Mucosal Specimens is provided courtesy of the NIH AIDS Research and Reference Reagent Program
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USA

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Appendix B: Source of Supplies
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Appendix D: Discontinued Collection Methods
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  - Collection of Pre-ejaculatory Fluid (Swab)
  - Collection of Rectal Secretions (Sno-Strips)
INSTRUCTIONS FOR SHIPMENT

1) Ship specimens to the address printed on the front cover.

2) Notify the Contact Person prior to shipment by FedEx. State the carrier and expected date and hour of arrival.

3) Bulk ship all specimens, routinely collected and frozen at –70°C, packed in dry ice on a monthly schedule directly to the MIL. Make these shipments on the same Monday of each month. [Each site should choose a Monday (1st, 2nd, 3rd or 4th of the month) and always ship on that same Monday each month.] If Monday is a holiday, ship on Tuesday.

4) Ship all specimens which cannot be frozen (e.g., for ELISPOT assays) immediately after collection so that they reach the MIL Tuesday-Friday. If Friday or weekend collection is absolutely unavoidable, discuss in advance with MIL to be sure that personnel will be available and able to perform the assays.

SPECIMEN LABELING

Each specimen must be labeled with the AVEG volunteer ID number, visit number, and visit date, as usual. For labeling consistency, a 5-letter code has been assigned for each specimen type and is to be included with the other identifying information on each label. These codes are:

### LISTING OF SPECIMEN TYPES FOR MUCOSAL ASSAYS

<table>
<thead>
<tr>
<th>SPECIMEN NAME</th>
<th>ABBREVIATED NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOOD IN ACDA*</td>
<td>ACDA</td>
</tr>
<tr>
<td>ACD PLASMA*</td>
<td>ACDPL</td>
</tr>
<tr>
<td>CERVICAL SECRETIONS/ASPIRATED</td>
<td>CRSAP</td>
</tr>
<tr>
<td>CERVICAL SECRETIONS/WICKED (Sno-Strip)*</td>
<td>CRSWK</td>
</tr>
<tr>
<td>CERVICAL SECRETIONS/WICKED (Polyfiltronics)</td>
<td>CSPWK</td>
</tr>
<tr>
<td>COLOSTRUM</td>
<td>COLOS</td>
</tr>
<tr>
<td>BLOOD IN EDTA*</td>
<td>EDTA</td>
</tr>
<tr>
<td>EDTA PLASMA*</td>
<td>EDTPL</td>
</tr>
<tr>
<td>FECES*</td>
<td>FECES</td>
</tr>
<tr>
<td>HEPARINIZED BLOOD/CORD</td>
<td>HEPBC</td>
</tr>
<tr>
<td>HEPARINIZED BLOOD/PERIPHERAL</td>
<td>HEPBP</td>
</tr>
<tr>
<td>HEPARINIZED PLASMA*</td>
<td>HEPPL</td>
</tr>
<tr>
<td>INTESTINAL WASH</td>
<td>IWASH</td>
</tr>
<tr>
<td>NASAL WASH</td>
<td>NWASH</td>
</tr>
</tbody>
</table>
**LISTING OF SPECIMEN TYPES FOR MUCOSAL ASSAYS continued**

<table>
<thead>
<tr>
<th>SPECIMEN NAME</th>
<th>ABBREVIATED NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAROTID SALIVA</td>
<td>PAROT</td>
</tr>
<tr>
<td>ACD PBMC*</td>
<td>PBACD</td>
</tr>
<tr>
<td>CITRATE PBMC*</td>
<td>PBCIT</td>
</tr>
<tr>
<td>HEPARINIZED PBMC*</td>
<td>PBHEP</td>
</tr>
<tr>
<td>PRE-EJACULATE SWABBED*</td>
<td>PJACS</td>
</tr>
<tr>
<td>PRE-EJACULATE WICKED (Snow-Strip)</td>
<td>PJACW</td>
</tr>
<tr>
<td>PRE-EJACULATE FLUID</td>
<td>PJACF</td>
</tr>
<tr>
<td>OTHER PLASMA*</td>
<td>PLSMA</td>
</tr>
<tr>
<td>RECTAL WASH</td>
<td>RWASH</td>
</tr>
<tr>
<td>RECTAL WICK (Sno-Strip)*</td>
<td>RWICK</td>
</tr>
<tr>
<td>RECTAL WICK (Polyfiltronics)</td>
<td>RPWCK</td>
</tr>
<tr>
<td>SEMEN</td>
<td>SEMEN</td>
</tr>
<tr>
<td>SEMINAL PLASMA</td>
<td>SEMPL</td>
</tr>
<tr>
<td>SERUM</td>
<td>SERUM</td>
</tr>
<tr>
<td>TEARS</td>
<td>TEARS</td>
</tr>
<tr>
<td>VAGINAL WASH</td>
<td>VWASH</td>
</tr>
<tr>
<td>WHOLE SALIVA</td>
<td>WSALV</td>
</tr>
</tbody>
</table>

* Not currently in use

**MUCOSAL SPECIMENS TRANSMITTAL FORM**

The Mucosal Specimens Transmittal Form (Appendix C) must be completed for each specimen shipped directly to the MIL. The form should be filed in the volunteer chart and a copy forwarded to the MIL with the shipment. Check off the appropriate specimen type and complete any additional specimen-specific information that is required. The additional information required for the specimen types listed below is essential for proper assaying of the specimens and analysis of the results. THIS INFORMATION MUST BE PROVIDED WITH THE SPECIMEN and entered into the Direct Shipment Database.

*All tubes or vials to be shipped must be sealed to insure against leakage.*

All samples which cannot be frozen should be shipped to UAB on the day of collection. For samples requiring isolation of viable cells, the Priority Overnight option must be used in order to receive the blood by 10:30 AM on the day after it is collected. If specimens must be shipped on Friday, notify the MIL well in advance to arrange for receiving and handling (isolation of cells and performance of the ELISPOT) on Saturday. The vaccine centers should take care to ship the blood in insulated containers during periods of extreme heat or cold.
The following table provides an overview of the shipping and aliquotting requirements for each specimen type. Please refer to each specific collection procedure for detailed instructions. To decrease the number of freeze/thaw cycles, a minimum number of aliquots are required for certain specimen types regardless of the total volume collected. For example, if 0.4 mL of parotid saliva are collected, four 0.1 mL aliquots should be made rather than two 0.2 mL aliquots or one 0.4 mL aliquot. It should be emphasized that 0.1mL is the absolute minimum acceptable volume, and accurate pipetting is essential. For example, do not place 80 µL into an aliquot which is labeled as 0.1 mL. If a large volume is collected, make as many aliquots as possible using the upper value of the volume/aliquot range given.
## SAMPLE SHIPMENT

<table>
<thead>
<tr>
<th>SPECIMEN TYPE</th>
<th>ALIQUOT REQUIREMENTS</th>
<th>SHIPMENT CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NUMBER OF ALIQUOTS</td>
<td>VOLUME/ALIQUOT</td>
</tr>
<tr>
<td></td>
<td>(minimum)</td>
<td>(mL)</td>
</tr>
<tr>
<td>BLOOD (PERIPHERAL/CORD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparinized</td>
<td>2**</td>
<td>10.0</td>
</tr>
<tr>
<td>Serum</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>CELLULAR SPECIMENS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue biopsies, mucosal swabs, cervical scrapings, semen cell pellets, etc.</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>COLOSTRUM</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>FEMALE GENITAL SECRETIONS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal Washings</td>
<td>4</td>
<td>0.4</td>
</tr>
<tr>
<td>Cervical Secretions (Wick)</td>
<td>2</td>
<td>0.5</td>
</tr>
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<td>Cervical Secretions (Aspirated)</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>MALE GENITAL SECRETIONS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-ejaculate (Sno-Strip)</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>Pre-ejaculate (Fluid)</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>Seminal Plasma</td>
<td>5</td>
<td>0.2 - 0.4</td>
</tr>
<tr>
<td>NASAL SECRETIONS</td>
<td>5</td>
<td>0.5 - 1.0</td>
</tr>
<tr>
<td>RECTAL SECRETIONS (Wick)</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>RECTAL SECRETIONS (Wash)</td>
<td>5 *</td>
<td>1.0</td>
</tr>
<tr>
<td>SALIVA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parotid</td>
<td>4</td>
<td>0.1 - 0.4</td>
</tr>
<tr>
<td>Whole</td>
<td>4</td>
<td>0.1 - 0.4</td>
</tr>
<tr>
<td>TEARS</td>
<td>2</td>
<td>0.1 - 0.4</td>
</tr>
</tbody>
</table>

Note: Instructions for processing and freezing are given under individual secretions.

* Store remainder of sample in large cryovials or 50 mL conical centrifuge tubes at site if desired.

** Minimum number of tubes depends on the protocol.
INTRODUCTION

Appropriate collection and processing of specimens is of paramount importance for subsequent evaluation of immune responses of vaccinees. Although the collection, processing and storage of serum specimens for further analyses do not pose special problems, external secretions will require special attention and coordinated effort. Because there are no universally accepted methods for collection of most external secretions, this manual detailing collection, processing, storage, and shipment of various external secretions, is intended for personnel at AVEUs involved in the collection of such specimens in order to ensure uniformity of collection procedures to allow for subsequent evaluation of immune responses induced by HIV vaccines at various centers. Appendix B provides the vendors for needed supplies. A videotape illustrating the collection of many of the mucosal specimens will also be provided.

STANDARD PROTOCOLS FOR THE COLLECTION, PROCESSING, AND STORAGE OF MUCOSAL SPECIMENS AT THE AVEUs

Rationale

Antibodies present in human external secretions are derived from two sources: plasma and local lymphoid cells distributed in mucosal tissues. The relative contribution of antibodies from these sources differs in individual secretions and is also influenced by the local conditions in the mucosal tissues. For example, antibodies found in whole saliva collected from individuals with healthy periodontia are mostly produced by plasma cells distributed in minor and major salivary glands. However, in patients with various stages of periodontal disease (i.e., the majority of middle-aged individuals), plasma-derived antibody contributes more significant quantities due to seepage from the crevicular fluid. Therefore, collection of parotid rather than whole saliva is essential in the evaluation of mucosal, secretory immunoglobulin A (S-IgA)-mediated immunity. In the female genital tract, large amounts of Ig are derived from blood during menstruation. Furthermore, hormonal status and the presence of local inflammation in mucosal tissues also significantly influence the relative contribution of Ig from the circulation. Thus, it is essential to measure not only the levels of S-IgA but also of IgG and albumin (derived mostly from plasma) to obtain a general view of the relative contribution plasma-derived versus locally produced Ig to the Ig pool found in given external secretions.

NOTE: Collection priorities are specific to each protocol.
SALIVA

Rationale

Saliva is the easiest external secretion to collect in humans. More importantly, it is one of the most representative fluids of all external secretions, because it contains low levels of IgG, especially in parotid secretions. Thus, we strongly emphasize that parotid saliva should be examined in all immunization studies to provide a comprehensive view of the effectiveness of systemically or mucosally administered vaccines for stimulation of S-IgA-mediated mucosal immune responses.

COLLECTION OF PAROTID SALIVA  [Label = PAROT]

Supplies:
- Intraoral cup (Schaeffer cup)
- Sterile transfer pipette
- Centrifuge tube
- Cryovials, 0.5 mL
- Petri dish

Although many collection devices (e.g., Curby cup) have been used to collect this fluid, we developed an intraoral plastic cup which is inexpensive, easily sterilized, disposable, easy to handle, and allows for the collection of several mL of pure parotid secretions. AVEUs will be provided with these cups upon request from DAIDS. The cups will be provided sterile and should be discarded after a single use.

Salivary stimulation (lemon juice, etc.) is discouraged.

Use of the Intraoral Cup

1) Parotid saliva collection is made with the intraoral cup by selecting the largest cup that will fit in the buccal vestibule comfortably. The cup is held between the thumb and forefinger of one hand with the flat surface of the cup toward the cheek surface where it is to be placed. The opening is oriented so that the straight cut is parallel to the floor and the opening is in the superior position.

2) The forefinger of the other hand is used to gently retract the corner of the mouth on the side to be sampled, and the cup is slid into the cheek with the round side next to the teeth and the opening in the flat side covering Stensen's duct of the parotid gland. Usually, the correct size cup will automatically cause the opening to fall immediately over the duct orifice. It is simple to check this position of the cup, since the plastic is clear and the orifice can be visualized easily, with good light. Gentle pressure on the external surface of the cheek over the cup will cause a small amount of air to be expressed from the cup and create a slight negative pressure that helps to keep the cup in place.

3) If collection is desired from both glands simultaneously, the procedure of the cup placement is repeated for the other cheek. A visual inspection may be made to determine when a sufficient
quantity for laboratory assay has accumulated in the cup, depending on the study being conducted.

4) Removal of the cup with the sample inside is a reverse procedure to that of placement. The corner of the mouth is gently retracted with the forefinger of one hand, while the thumb and forefinger of the opposite hand are used to carefully grasp the forward edge of the cup. The cup is moved out of the mouth, keeping it in the same relative position it occupied in the vestibule (upright), with the flat edge of the opening parallel to the floor.

5) When clear of the lips, the cup is turned with the round side down and the opening face up. In this position, the contents will not spill, and it may be set down while the second cup is removed in the same manner. While removing the cup from the mouth, some investigators recommend the placement of a petri dish under the cup to prevent spillage.

6) The sample in each cup is transferred with a pipette to a graduated centrifuge tube on ice. Discard the cup and immediately prepare a minimum of four aliquots (0.1 - 0.4 mL) into cryovials (0.5 mL). Freeze at \(-70^\circ\text{C}\) and ship on dry ice.


**COLLECTION OF WHOLE SALIVA**  [Label = WSALV]

Although easily collected, whole saliva is not the best representative external secretion because of frequent admixture of plasma-derived proteins, including immunoglobulins, due to periodontal inflammation and seepage of gingival crevicular fluid which occurs with variable intensity in almost all adults. The crevicular fluid contribution is likely to increase with chewing. Furthermore, whole saliva is viscous and more difficult to handle than parotid saliva.

This fluid will be collected using a commercially available device (Salivette).

**Supplies:**
- Salivette tube
- Cryovials, 0.5 mL
- Table-top centrifuge
- Protease inhibitor solution (as specified in Appendix A)
- NaN₃

1) The cylindrical shaped swab is removed from the insert and placed in the mouth.

2) The swab is chewed for 30 - 45 sec or until one can no longer prevent swallowing the saliva produced. If the swab cannot be chewed, it can be placed under the tongue for 30 - 45 sec.

3) After the above procedure is complete, the swab is returned to the insert and the Salivette firmly closed with the stopper.
4) The Salivette is centrifuged for 2 min at 1,000 x g. Higher g forces result in only slightly higher yields of saliva. During centrifugation, the saliva will pass from the cylindrical shaped swab through the hole in the bottom of the suspended tube into the clear centrifuge tube. Mucous strands and particles will be caught in the conical tip of the centrifuge tube allowing easy decanting of the clear saliva.

5) Immediately add protease inhibitor solution (Appendix A) and NaN₃. Prepare a minimum of four aliquots (0.1 - 0.4 mL) into cryovials (0.5 mL capacity). Freeze immediately at −70°C and ship on dry ice.
INTESTINAL SECRETIONS

Rationale

Human intestinal secretions contain antibodies against enteric bacteria and food antigens as well as against antigens introduced by immunization, especially through mucosal routes. Because of HIV transmission through anal intercourse, antibodies locally produced in the lower intestines are potentially important. Therefore, it is proposed to determine levels of HIV-specific antibodies in intestinal secretions induced by intrarectal, oral, or systemic administration of antigens.

The collection procedures described do not include collection of feces due to the limited information obtained from the fecal extracts.

COLLECTION OF INTESTINAL SECRETIONS (INTESTINAL WASH) [Label = IWASH]

Although this method of collection provides the best samples for analysis of mucosal immune responses (the collected fluid contains secretions of both small and large intestines), there are several reasons that it is not the method of choice: the amount of volunteer time required, discomfort to the volunteer resulting in low compliance, and the lack of suitable facilities in some centers.

Supplies:
- Golytely (buffered PEG solution)
- Plastic collection device ("hat")
- Cheesecloth
- Large funnel, 115 mm
- Centrifuge tubes, 50 mL
- Soybean trypsin inhibitor (1.0 mg/mL in PBS)
- EDTA (1M in PBS)
- Phenylmethylsulfonyl fluoride in 95% ethanol (PMSF)
- NaN₃ (20 mg/mL in PBS)
- Sterile filter, 150 mL/0.45 µm
- Fetal calf serum
- Cryovials, 1.5 mL
- Table-top centrifuge

1) Ask volunteer to drink 2 liters of Golytely within a period of 60 - 90 min.

2) After the stool becomes watery, collect approximately 200 - 400 mL of the effluent using a plastic collection device (known as a "hat") placed over a commode.

3) Filter the secretions through cheesecloth taped on a large funnel into a 50 mL centrifuge tube. Add soybean trypsin inhibitor (1.0 mg/mL in PBS) and EDTA (1 M in PBS), into each tube to a final concentration of 10% v/v and 5% v/v respectively, and then centrifuge the tube at 550 x g for 10 min.

4) Transfer the supernatant to a clean centrifuge tube. Add a solution of 100 mM phenylmethylsulfonyl fluoride (PMSF) in 95% ethanol to a final concentration of 1% v/v. Centrifuge the tubes at 2,200 x g for 30 min.
5) Transfer the supernatant to a clean tube. Add both PMSF and 20 mg/mL sodium azide to a concentration of 1% v/v, followed 15 min later by fetal calf serum at a final concentration of 4% v/v as a competitive substrate for any remaining protease. Filter the secretions through a 150 mL/0.45 µm sterile filter into a test tube on ice. Aliquot (1 mL) immediately into cryovials (1.5 mL). Freeze at \(-70^\circ\text{C}\) and ship on dry ice.

**COLLECTION OF RECTAL SECRETIONS (POLYFILTRONICS WICK)** [Label = RPWCK]

This procedure using polycarbonate wicks has been developed for the collection of intestinal and genital tract secretions of experimental animals and later applied to humans. Although relatively simple and rapid, only small volumes of local secretions, frequently contaminated by plasma proteins or blood due to the damage of mucosa, are obtained by this method.

This method, which does not require anoscopy, employs a wick from Polyfiltronics. Its diameter is 8.5 mm, length as manufactured is 61.24 mm.

**Supplies:**
- Elution media (See Appendix A)
- Polyfiltronics wick applicator (See Appendix A)
- Lubricating jelly
- Conical centrifuge tube, 15 mL (with screw-cap)
- Sterile syringe filters, 0.45 µm
- Ice bucket and ice
- Scale
- Table-top centrifuge

1) Prepare elution media (see Appendix A).

2) Prepare Polyfiltronics wick applicator (see Appendix A).

3) Lubricate the applicator with lubricating jelly. With patient lying on his or her side, insert the applicator into the anus. Maintain control of the syringe plunger and pull back the barrel of the applicator. The string will remain accessible through the anus for subsequent removal of the wick.

4) Leave the wick in place for 5 minutes.

5) Remove the wick by gently pulling on attached string. Place immediately in 15 mL polypropylene screw-cap conical centrifuge tube and obtain "wet weight" on a scale accurate to at least .01 g. Use the same 15 mL conical tube in which the wick was weighed previously, and write the new weight on the outside of the tube. From this value, the volume of rectal secretion absorbed by the wick is determined.

6) Immediately proceed to elute the wick. Add 5 mL of elution media. Let soak 30 minutes on ice. The wick will absorb most of this volume.
7) Suspend wick by string within conical tube. Tighten the cap over the strings. The bottom of the wick should be above the 5 mL mark of the tube.

8) Centrifuge the wick at 2000 x g for 10 minutes at 4°C. Most of the PBS/mucosal sample will spin out of wick.

9) Filter the eluted fluid through a 0.45 µm filter. Aliquot and freeze at -70°C.

**COLLECTION OF RECTAL SECRETIONS (RECTAL WASH)** [Label = RWASH]

This procedure is well-accepted by the donor as well as the staff involved. It yields adequate volumes of fluids for subsequent assays. The problems encountered in using absorbent materials which may damage the rectal mucosa (discussed above) are circumvented. However, the secretions collected are representative of local immunoglobin production and do not reflect the immune status of the entire intestine.

**Supplies:**
- 60 mL luer-lock syringes (2)
- Graduated transfer pipette, 1 mL
- Sterile saline, 100 mL (room temperature)
- Collection device ("hat")
- Lubricating jelly
- Conical centrifuge tubes, 50 mL, (2)
- Centrifuge tubes (for 10,000 x g)
- Sterile filters, 150 mL/0.45 µm
- Cryovials, 1.5 mL
- Ice bucket with ice
- Table-top centrifuge
- Vacuum filter device

1) Fill two 60-mL syringes with 50 mL saline each. Place the cup tip of a disposable, graduated 1-mL transfer pipette onto the hub of the syringe. Those pipettes are made of flexible polypropylene, allowing a good balance between the proper rigidity for insertion and flexibility for limiting discomfort. Cut the bulb from the barrel of the pipette, and fit the cut end snugly over the syringe.

2) Place the volunteer in the side-lying position.
3) Lubricate the syringe tip and anus lightly with lubricating jelly. Insert the syringe gently into the rectum. Slowly depress the plunger to instill the saline into the rectum.

4) Repeat this procedure with the second syringe of saline.

5) Ask the volunteer to retain the saline for 5 minutes if possible, then expel the saline into the collection device or "hat" (see intestinal wash procedure, above).

6) Transfer specimens to 50-mL conical tubes and place on ice. Transport the tubes, on ice, to the laboratory for immediate processing.

7) Clarify the secretions by centrifugation at 2,000 x g for 10 minutes at 4°C in a tabletop centrifuge (i.e. 3000 rpm in a Beckman GH 3.7 rotor or comparable device). This procedure removes gross fecal debris and some mucus.

8) Transfer the supernatant into a centrifuge tube capable of centrifugation at 10,000 x g. Centrifuge at 10,000 x g at 4°C. Determining the right speed to reach this g-force depends upon the particular rotor used. Any laboratory capable of preparing plasmid DNA preparations will have such a centrifuge/rotor combination for this step. For example, if the centrifuge is a Sorvall RC-5B, and the speed using the SA-600 rotor to reach 10,000 x g is 10,000 rpm. This step removes remaining mucus and debris. Although some rectal specimens can be filtered without this higher-speed centrifugation step, many will clog the filter if it is omitted.

9) Decant supernatant into 50 mL conical tubes. Add protease inhibitors and sodium azide (0.02%).

10) Filter liquid through a 150 mL/0.45 µm filter. Given the volumes involved, this is easiest with a 100 or 150 mL vacuum filter device. 0.45 µm syringe filters may be substituted, but they tend to clog and are less convenient.

11) Aliquot the filtered samples into cryovials and freeze at -70°C.
FEMALE GENITAL TRACT SECRETIONS

Rationale

The collection procedures described provide samples suitable for immunological analysis and are not intended for virological studies. Female genital tract secretions contain locally-produced and plasma-derived Ig. Depending on the day of collection during the menstrual cycle, the levels and properties of Ig are significantly different. This fact should be considered in evaluation of immune responses in female genital tract secretions and the collections should be performed at the same time during the individual menstrual cycles. We have determined that the optimal time of collection of both vaginal washings and cervical fluids is at midcycle. At this time, the quantity of cervical mucus is at the highest level. For women with regular cyclic menses, this can be estimated to be cycle day 14. For those with irregular menses, a convenient method for determination of the midcycle would be with urinary LH test kits which can be given to the volunteer who can be instructed to report for collection the morning after the urinary LH surge.

The volume of cervical mucus can range from 200-800 µL when collected at the time of ovulation. Samples collected in the proliferative phase of the menstrual cycle during the first week are contaminated with menstrual blood; this will complicate any interpretation of secretory immune products. Additionally, the volume of the cervical mucus between days 8 and 10 is rather low (between 20 and 100 µL, and often proves inadequate for extensive characterization). Similarly, after ovulation when endogenous progesterone production increases, the cervical mucus becomes more viscous and the volume decreases. This cervical mucus also is difficult to work with and has not been satisfactory for many studies.

Women who use birth control pills as their method of contraception are likely to be included in these studies. In this case, the vaginal washings and cervical mucus should be collected on day 14, after the onset of the menstrual cycle. Specify the birth control pill prescribed. It is also possible that some women involved in these studies will have undergone hysterectomy. Please indicate on specimen collection form.

The reason for collection on day 14 with or without ovariectomy (please specify) after the onset of the menstrual cycle is that the immunization and collection protocols for each volunteer should be discussed, in advance, among responsible investigators at the AVEUs and the MIL to determine optimal timing. As an example, we propose the following schedule:

<table>
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<th>14 DAYS</th>
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<th>28 DAYS</th>
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<td>IMMUNIZATION</td>
<td>(POST-LMP)</td>
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±2 days of ovulation
Collection of cervical secretions
Vaginal washing
Parotid saliva
Blood (for ASC and specific antibodies)

Collection and Processing of Specimens

Collection of blood for ASC (7 days post-immunization)

When a protocol specifies both a vaginal wash and collection of cervical secretions, the wash should be performed first.

ELIGIBILITY DETERMINATION FOR CERVICO-VAGINAL SECRETION COLLECTION

Volunteers should be assessed for exclusion criteria prior to collection.

Supplies:
- OvuQuick, urinary LH test kit
- Sema test kit
- Hemoccult test kit

Reasons for Exclusion
a) Abnormal vaginal discharge
b) Sexual intercourse without the use of condom (ejaculate was deposited into vagina) within 72 hr prior to collection. (A kit which will detect seminal fluid in cervico-vaginal fluids up to 24 hrs post coitus is the Sema kit)
c) Douching within 72 hrs prior to collection
d) Blood contamination (Specimens can be assessed for blood contamination with the Hemoccult test)

COLLECTION OF VAGINAL WASHINGS [Label = VWASH]

Supplies:
- Sterile saline, 3 mL (room temperature)
- Sterile plastic transfer pipette
- Round bottom plastic tube (12x75 mm with caps)
- Cryovial, 1.5 mL
- Protease inhibitor solution (see Appendix A)
- NaN₃
- Sterile vaginal speculum

1) Assist the volunteer to assume the supine position with feet placed in stirrups. Direct light to the perineal area and place a sterile speculum into the vaginal vault.

2) Put 3 mL sterile saline into a sterile plastic transfer pipette and vigorously flush it several times over the cervix and around the external cervical os. Do not use larger volumes of saline; this dilutes the immunoglobulins which tend to aggregate. Collect this material with the same pipette specified above and place it into a test tube on ice. Prepare aliquots (0.4 mL) into cryovials (1.5 mL capacity) as soon as possible.

3) Add protease inhibitor solution and NaN₃ at one-tenth the specimen volume.
4) Cap and label the cryovials. Freeze at \(-70^\circ C\) and ship on dry ice.

**PROTOCOLS 401 and 402 (involving HIV-infected volunteers) only:** Proceed as described in steps 1 and 2, except: when the specimen is collected, place it in a test tube and centrifuge in microfuge tubes for 5 min at high speed. Collect the supernatant, label appropriately and freeze. The sediment at the bottom should also be frozen at \(-70^\circ C\), labeled, and both test tubes should be shipped to UAB on dry ice. The aliquotting of the supernatant should be done as described in step 2. The pellet should be shipped in a single test tube.

**COLLECTION OF CERVICAL SECRETIONS (ASPIRATE)** [Label = CRSAP]

**Supplies:**
- Unimar aspirette
- Cryovials, 0.5 mL
- Sterile vaginal speculum
- Ice bucket and ice

1) Assist the volunteer to assume the supine position with feet placed in stirrups. Direct light to the perineal area and place a sterile or disposable speculum into the vaginal vault.

2) Collect cervical mucus by inserting an aspirette into the cervix. The aspirette is a symmetrical tube (diameter approximately 3 mm, graded in 1 cm increments) with a Teflon plunger. Insert the aspirette 2 cm into the uterine os. Measure and dispense the collected cervical mucus into a test tube on ice. Make a minimum of two aliquots (0.1 - 0.2 mL) into small cryovials as soon as possible.

3) Cap and label cryovials. Freeze immediately at \(-70^\circ C\) and ship on dry ice.

**COLLECTION OF CERVICO-VAGINAL SECRETIONS (POLYFiltrONICS WICK)** [Label = CSPWK]

**Supplies:**
- Dressing Forceps-10”
- Polyfiltronics wick applicator (see Appendix A)
- Elution media (see Appendix A)
- Lubricating jelly
- Conical centrifuge tube, 15 mL
- Sterile filter, 150 mL/0.45 µm
- Ice bucket and ice
- Table-top centrifuge
- Scale

1) Prepare elution media (see Appendix A).

2) Prepare the Polyfiltronics wick applicator (see Appendix A).

3) Instruct the donor regarding procedures for self-insertion of the applicator, placing as high in the vagina as possible without causing discomfort.
4) Instruct the donor to leave the wick in place for 5 minutes.

5) Remove the wick using the attached string and place immediately in the same conical tube and obtain "wet weight" on a scale accurate to at least 0.01 g. Do not remove the string. Write the new weight on the outside of the tube. From this value, the volume of cervico-vaginal secretion absorbed by the wick is determined.

6) To elute the wick, thaw and add elution buffer containing protease inhibitors (see Appendix A).

7) Leave on ice for 30 minutes. Suspend wick by string within the conical tube. Tighten the cap over the strings. The bottom of the wick should be above the 5 mL mark of the tube.

8) Centrifuge the wick at 2,000 x g for 10 minutes at 4°C.

9) Filter the eluted fluid through a 150 mL/0.45 µm filter. Aliquot into cryovials and freeze at -70°C.
Male Genital Tract Secretions

Rationale

Although human seminal plasma contains Ig, including IgA, the origin has not been conclusively established. Some investigators find IgA-secreting cells and secretory component positive (SC⁺) epithelial cells in the prostate gland while others do not. Pre-ejaculate urethral secretions are produced by small glands in the penile urethra ("Glands of Litre") during sexual excitement, and serve as lubricating fluid for sexual intercourse. They have been shown to contain IgA. Although the pre-ejaculatory fluid is highly viscous and low volumes are collected, this secretion usually contains higher levels of immunoglobulins than does seminal fluid.

Reasons for Exclusion
a) Symptoms of infection (e.g., abnormal penile discharge or burning with urination, because they may be contaminated with plasma-derived Ig due to inflammatory transudation).
b) Semen samples do not liquify (and should be discarded).
c) Any ejaculation within 48 hr prior to collection.

Collection of Semen [Label = SEMEN]

Supplies:
- Sterile specimen collection cup (4 oz, with screw-on lid)
- Dulbecco’s phosphate-buffered saline (PBS)
- Protease inhibitor solution (see Appendix A)
- NaN₃
- Cryovials, 0.5 mL
- Conical centrifuge tube, 15 mL
- Table-top centrifuge

This procedure is commonly used for examination of semen from patients for potential infertility, and also for donation of sperm to a sperm bank.

1) Ask male volunteer to abstain from ejaculation for 48 hr prior to the designated day of collection.

2) Give volunteers a sterile specimen container. Ask the volunteer to wash his hands and genitals with soap and warm water prior to masturbation. No lubricant or condom should be used, and remind the volunteer that the semen should not be admixed with saliva. The semen collection should preferably be made during the clinic visit in a private collection room. If semen is collected at home (in a specimen container furnished by the AVEU), it should be brought to the AVEU immediately. (It will be helpful to provide the volunteer with an insulated container for transport.)

3) Refrigerate ejaculate (4°C); it will spontaneously agglutinate, but will become liquid upon standing for 1 hr in the refrigerator.

4) To obtain seminal plasma for antibody measurement, dilute semen with an equal volume of PBS and centrifuge at 1,200g/10 min. Add protease inhibitor solution and NaN₃ at one tenth
Collection and Processing of Specimens

specimen volume. Prepare a minimum of five aliquots (0.2 - 0.4 mL) of the supernatant in cryovials (0.4 mL). Freeze immediately at -70°C and ship on dry ice.

PROTOCOLS 401 and 402 (involving HIV-infected volunteers) only: Proceed as described in steps 1-3. After centrifugation at high speed, collect the supernatant, aliquot, freeze at -70°C, and ship on dry ice. The cell pellet should also be frozen at -70°C and shipped on dry ice to UAB. All other cell pellets should be discarded.

COLLECTION OF PRE-EJACULATORY FLUID [Label = PJACF]

Initial data concerning the levels of immunoglobulins in this fluid collected by different methods indicate that the use of wicks or swabs with subsequent elution (and therefore, dilution) is not as informative as the direct collection of pre-ejaculate fluid.

Supplies:
- Cryovials, 0.5 mL

Specific instructions to be given to the donor for collection of pre-ejaculate

1) Abstain from ejaculation for a minimum of 48 hrs.

2) Wash hands and glans penis with mild soap and water, rinse well and dry with a clean towel.

3) Proceed to masturbate, but do not ejaculate.

4) During this process, pre-ejaculatory fluid generally appears as small droplets at the tip of the penis following sexual arousal. When pre-ejaculate fluid appears, place the edge of the cryovial to the tip of penis. May be repeated if new drops appear.

5) Keep in the refrigerator and deliver pre-ejaculate (and semen) specimens to the laboratory, as soon as possible.

6) In the lab, freeze the pre-ejaculate at -70°C and ship on dry ice to MIL.

COLLECTION OF PRE-EJACULATORY FLUID (SNO-STRIP) [Label = PJACW]

Supplies:
- Sno-Strips
- Eppendorf tubes (screw cap)
- Cryovials 0.5 mL
- Dulbecco’s phosphate-buffered saline (PBS)
- Bovine serum albumin

1) Ask the donor to collect as much pre-ejaculatory fluid as possible onto the Sno-Strip. Then, place the Sno-Strip into the tube, close the cap, refrigerate or place on ice, and deliver it to the clinic in an insulated container, along with the semen sample.
2) Add PBS containing 1% BSA (500 µL) directly to the tube with the Sno-Strip, vortex for 1 min and store at −70°C. Ship on dry ice to the MIL.
NASAL SECRETIONS  [Label = NWASH]

Supplies:
- Sterile pipette, 5 mL or syringe, 10 mL
- Sterile saline solution
- Sterile specimen collection cup (4 oz, with screw-on lid)
- Cryovials, 1.5 mL
- Ice bucket and ice

1) Have the subject sit in a chair with back support, with head tilted back. Have the volunteer make a clicking or "K-K-K" sound to close off the glottis so as not to swallow the saline or choke.

2) Instill with a sterile plastic pipette or plastic syringe 2.5 mL of sterile saline kept at room temperature or pre-warmed to 37°C into each nostril (each nostril can be lavaged separately). Instruct the subject to hold his/her breath and not to swallow, keeping the saline solution in the nose for 10-30 sec.

3) Have the volunteer tilt head forward, placing a sterile collection cup under the nose, and then blow the nose into the cup.

4) Put the cup on ice and aliquot (0.5 - 1.0 mL) in cryovials (1.5 mL capacity) within 15 minutes. Freeze at −70°C and ship on dry ice.
**COLOSTRUM** [Label = COLOS]

Collection of colostrum should be performed at the hospital 1 - 3 days after delivery. The earliest possible collection is desirable because IgA content is highest and fat content is lowest. Donors who do not intend to breast feed should be excluded from multiple collections to prevent the onset of lactation.

**Reasons for Exclusion**

Mastitis, inflammation of nipples, fissures or cracks (rhagades) of the nipples which result in contamination of colostrum with blood.

**COLLECTION OF COLOSTRUM**

**Supplies:**
- Latex gloves
- Alcohol swabs
- Sterile gauze pads
- Sterile conical centrifuge tubes, 50 mL
- Cryovials, 1.5 mL

1) Wearing sterile latex gloves, clean the nipple with an alcohol swab or moistened sterile gauze pad. Wipe dry with sterile gauze.

2) Using gentle pressure applied repeatedly on the nipple, express colostrum directly into a sterile 50-mL centrifuge tube. The amount of colostrum obtained from individual donors varies considerably, but it should be possible to collect more than 1 mL in a few minutes. A disposable breast pump may also be used to collect the specimen.

3) When collection is complete, blot the nipple with sterile gauze to dry.

4) Do not aliquot the specimen. Transfer to cryovials. Freeze at −70°C and ship on dry ice.
**TEARS** [Label = TEARS]

To stimulate tear flow, inhalation of chopped onion vapors or ammonia has been used. However, a method originally described by Fleming is recommended because, in a short collection time, it consistently provides volumes of tears amenable to immunological analyses.

**COLLECTION OF TEARS**

**Supplies:**
- Fresh orange
- Graduated transfer pipette
- Scalpel
- Cryovials, 0.5 mL
- Eppendorf tubes (with screw-caps)
- Visine

1) Wash fresh orange under tap water and dry. Cut a small piece of orange rind from the surface of the orange.

2) Squirt the mist of squeezed rind in the lateral corner of an opened eye.

3) Tilt the head down and collect tears with a pipette from the lateral side; transfer the tears into an Eppendorf tube. Up to 0.3 mL of tears can be collected from one eye.

4) At the end of collection, wash the eyelids with a wet towel and dry. Place repeatedly several drops of Visine or a similar ophthalmic solution into the conjunctival sac.

5) Prepare a minimum of two aliquots (0.1-0.4 mL) into cryovials (0.5 mL). Freeze at −70°C and ship on dry ice.
PERIPHERAL BLOOD AND CORD BLOOD FOR ELISPOT [Label = HEPBP and HEPBC]

Supplies:
- Vacutainer tubes, 10 mL green-stoppered
- Reusable ice
- Teri-Wipes

Depending on the protocol, blood should be collected in requested volumes of 10 mL heparinized (green-stoppered) tubes, from each volunteer at all designated time points. Blood should be shipped at ambient temperature packed in polystyrene containers protected from extreme heat and cold. Although the mononuclear fraction from peripheral blood can be isolated at individual AVEUs, slight differences in separation techniques (differences in centrifugal forces, collection of fractions from gradient, etc.) may result in inconsistent cell yield and composition of samples from different AVEUs. To avoid such variables, PBMC will be isolated at MIL. Because the ELISPOT assays must be performed immediately, do not ship samples on the weekend unless arrangements to do so have been made well in advance.

Priority Overnight option must be used, in order to receive the blood by 10:30 AM on the day after it is collected. It is our experience that the optimum recovery of cells will be from uncentrifuged, unrefrigerated blood, and that recovery of cells from blood within a day after collection is virtually as efficient as on the day of collection. Samples should be insulated from hot and cold temperatures.

OTHER CELLULAR SPECIMENS

Supplies:
- Polystyrene containers (for shipment)
- Reusable ice
- Teri-Wipes
- Rpmi 1640 or Hanks balanced salt solution, with fetal bovine serum, antibiotics, and anti-fungals added
- Eppendorf tubes (with screw-caps)

All specimens, including mucosal tissue biopsies, mucosal swabs, cervical scrapings, and semen, will be shipped to UAB packaged in well-isolated and sealed (with tape) polystyrene containers with REUSABLE ICE packages produced commercially by several companies. These packages are kept at −20°C and are added to the specimens, wrapped in Teri-Wipes to prevent their breakage, shaking, and freezing during shipment. Because the ELISPOT assay for antibody-forming cells and cytokine-secreting cells will be performed on such specimens, we cannot isolate and maintain cells from frozen samples. Genital swabs and scraping specimens, as well as biopsy tissues intended for isolation of cells to be analyzed by ELISPOT or immunofluorescence microscopy, will be collected and transported in RPMI 1640 or Hanks Balanced Salt Solution (HBSS) media to which the following will be added for each 500 mL: 50 mL fetal bovine serum, penicillin/streptomycin (5 mL) gentamicin (500 mL), and fungizone (125 mL). No protease inhibitors are required for these samples.
APPENDIX A

PREPARATION OF MATERIALS AND REAGENTS

Preparation of Elution Media

**Supplies:**
- Dulbecco’s phosphate-buffered solution (PBS)
- Fetal calf serum
- NaN₃ solution (sodium azide), 20 mg/mL

Add sodium azide to Dulbecco’s PBS to a concentration of 0.02% w/v. Add 10% fetal calf serum (v/v). This elution solution may be stored at 4°C for several weeks. When ready to elute, add the protease inhibitors from concentrated stocks as outlined below. For example, the ALA stock is 1000x, so 1 part ALA is diluted in 999 parts of elution buffer.

Preparation of Protease Inhibitor Solution

**Supplies:**
- PMSF, 100 mM solution in 95% ethanol
- Aprotinin, 5 mg/mL in H₂O
- Leupeptin, 1 mg/mL in H₂O
- Antipain, 1 mg/mL in H₂O
- Pepstatin, 1 mg/mL in methanol
- NaN₃ solution (sodium azide), 20 mg/mL

Prepare stock solutions:

The aprotinin, leupeptin, and antipain may be mixed in one tube at the final concentrations above. The resulting mix, a 1000X concentrate is referred to as ALA. The pepstatin stock is also 1000X. The 100 mM stock of PMSF is 100X. The sodium azide stock is also 100X. The final concentration employed in mucosal specimens is thus 5 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL antipain, 1 µg/mL pepstatin, 1 mM PMSF, and 0.02% NaN₃. ALA stock solutions in 1000x concentrate form can be stored for up to 3 months at -20°C, however, once diluted to 1x form, solution must be used immediately.

Preparation of Polyfiltronics Wick Applicator

**Supplies:**
- Plastic tampon applicator (regular size)
- Polyfiltronics wick, 5.5 cm
- Dental floss
- 3 cc syringe
- Conical centrifuge tube, 15 mL (with screw-cap)

1) Trim the length of the wick to 5.5 cm with a pair of scissors.
2) Cut a 35 cm length of dental floss; tie securely to the wick 1.5 cm from one end. Make the knot in the middle of the floss, so that there are two string ends of close to equal length.
3) Weigh the wicks and string individually within a 15 mL polypropylene conical tube (with cap) to obtain "dry weight". Write the dry weight on the conical tube. These weighed wicks can be prepared in advance of the volunteer visit.
APPENDIX A continued

4) Prior to collection of specimen, prepare the wick applicator as shown below. First, insert the wick into the tampon applicator. The end of the wick with the string tied to it should face the "plunger" end of the applicator (see diagram). A plunger removed from a 3 cc syringe is then placed in the end of the applicator.

![Diagram of wick applicator components: Polyfillronics wick with string (floss) attached, Tampon applicator, Plunger from 3cc syringe, Assembled wick applicator]
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# APPENDIX B
## SOURCE OF SUPPLIES

<table>
<thead>
<tr>
<th>ITEM</th>
<th>SOURCE</th>
<th>CATALOG #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent, PMSF</td>
<td>Boehringer Mannheim, 9115 Hague Rd, PO box 50414, Indianapolis, IN 46250-0414 Tel.# 800/262-1640</td>
<td>Cat.# 236608</td>
</tr>
<tr>
<td>Reagent, Sodium Azide</td>
<td>Sigma Tel.# 800/325-3010</td>
<td>Cat.# S2002</td>
</tr>
<tr>
<td>Reagent, Soybean Trypsin Inhibitor (0.1mg/mL in PBS)</td>
<td>Sigma Tel.# 800/325-3010</td>
<td>Cat.# T9003 (chromatography grade) Cat.# T9128 (crude grade)</td>
</tr>
<tr>
<td>Reagent, Strep Solution Pen</td>
<td>Gibco/BRL Tel.# 800/828-6686</td>
<td>Cat.# 12K9327</td>
</tr>
<tr>
<td>Saline, sterile solution</td>
<td>Abbott Lab Hosp Prod Div, 1 Abbott Park Rd, Abbott Park, IL 60064 Tel.# 800/222-6883</td>
<td>Cat.# D-R10</td>
</tr>
<tr>
<td>Scalpel</td>
<td>VWR Scientific Tel.# 800/932-5000</td>
<td>Cat.# 25853-003</td>
</tr>
<tr>
<td>Shipping container, polystyrene</td>
<td>Tech Pak Inc, Peabody, MA 01960 Tel.# 800/225-5019</td>
<td></td>
</tr>
<tr>
<td>Sno-Strips (62 x 6 mm, 5 mm at rounded collection tip)</td>
<td>Akorn Inc., 100 Akorn Drive, Abita Springs, LA 70420 Tel.#504/893-9300 or 800/535-7155</td>
<td>Cat.#1220</td>
</tr>
<tr>
<td>Swab, culturette</td>
<td>Fisher Tel.# 800/766-7000</td>
<td></td>
</tr>
<tr>
<td>Syringe (standard, 3 cc luer-lock)</td>
<td>Fisher Tel.# 800/766-7000</td>
<td>Cat.# 14-823-40</td>
</tr>
<tr>
<td>Syringe (standard, 10 cc luer-lock)</td>
<td>Fisher Tel.# 800/766-7000</td>
<td></td>
</tr>
<tr>
<td>Syringe (standard, 60 cc luer-lock)</td>
<td>Becton Dickinson</td>
<td>Cat.# BD309663</td>
</tr>
<tr>
<td>Teri-Wipes</td>
<td>Kimberly-Clark Corp, Roswell, GA 30076 Tel.# 800/633-0333</td>
<td>Cat.# C-6405-10</td>
</tr>
<tr>
<td>Tube, centrifuge (10,000 x g)</td>
<td>Nalgene, Nalge Nunc International, PO Box 20365, Rochester, NY 14602-0365 Tel.# 800/625-4327</td>
<td>Cat.# 3112 or 3113</td>
</tr>
<tr>
<td>Tube, centrifuge (screw-cap, 15 mL)</td>
<td>Fisher Tel.# 800/766-7000</td>
<td>Cat.# 07200485</td>
</tr>
<tr>
<td>Tube, Sterile centrifuge (50 mL)</td>
<td>Fisher Tel.# 800/766-7000</td>
<td>Cat.# 0553855</td>
</tr>
</tbody>
</table>
## APPENDIX B

### SOURCE OF SUPPLIES

<table>
<thead>
<tr>
<th>ITEM</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Tube, Eppendorf (screw-cap microcentrifuge tube, 1.5 mL polypropylene)</td>
<td>Sarstedt&lt;br&gt;Tel.# 800/257-5101</td>
<td>Cat.# 72-692</td>
</tr>
<tr>
<td>Tube, round bottom (12x75 mm plastic tube with caps)</td>
<td>Uri System Tubes, Becton-Dickinson Lab Ware, Lincoln, Park, NJ (Fisher)&lt;br&gt;Tel.# 800/766-7000</td>
<td>Cat.# 14-375-200&lt;br&gt;(tubes);&lt;br&gt;Cat.# 14-375-205&lt;br&gt;(caps)</td>
</tr>
<tr>
<td>Tube, Salivette</td>
<td>Sarstedt Co, NJ&lt;br&gt;Tel. # 800/257-5101</td>
<td>Cat.# 51-1534-002</td>
</tr>
<tr>
<td>Tube, Vacutainer (10 mL, green top)</td>
<td>Fisher&lt;br&gt;Tel.# 800/766-7000</td>
<td>Cat.# 02-685-3B</td>
</tr>
<tr>
<td>Vial, Cryovials (0.5 mL)</td>
<td>Sarstedt&lt;br&gt;Tel.# 800/257-5101</td>
<td>Cat.# 72-730-006</td>
</tr>
<tr>
<td>Vial, Cryovials (1.5 mL)</td>
<td>Sarstedt&lt;br&gt;Tel.# 800/257-5101</td>
<td>Cat.# 72-694-006</td>
</tr>
<tr>
<td>Visine</td>
<td>Polyfiltronics Groups Incorporated, 100 Weymouth Street, Rockland, MA 02370&lt;br&gt;Tel.# 617/878-1133</td>
<td>Cat.# UW 8.5 - 61.24&lt;br&gt;(pack of 100)</td>
</tr>
</tbody>
</table>
APPENDIX C

AIDS VACCINE EVALUATION GROUP

MUCOSAL SPECIMENS TRANSMITTAL FORM

VOLUNTEER ID NUMBER: ____________________________

VISIT DATE: _______ _______ _______

VISIT NUMBER: _______

PLEASE CHECK SPECIMEN(S) CONTAINED IN THIS SHIPMENT

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>ABBREVIATION</th>
<th>SPECIMEN</th>
<th>ABBREVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>____ BLOOD IN ACDA</td>
<td>ACDA</td>
<td>____ ACDC</td>
<td>PBACD</td>
</tr>
<tr>
<td>____ ACD PLASMA</td>
<td>ACDPL</td>
<td>____ CITRATE PBMC</td>
<td>PBCT</td>
</tr>
<tr>
<td>____ CERVICAL SECRETIONS/ASPIRATED*</td>
<td>CRSAP</td>
<td>____ HEPARINIZED PBMC</td>
<td>PBHEP</td>
</tr>
<tr>
<td>____ CERVICAL SECRETIONS/WICKED (Sno-Strip)*</td>
<td>CRSWK</td>
<td>____ PRE-EJACULATE SWABBED*</td>
<td>PJACS</td>
</tr>
<tr>
<td>____ CERVICAL SECRETIONS/WICKED (Polyfiltronics)*</td>
<td>CSPWK</td>
<td>____ PRE-EJACULATE WICKED*</td>
<td>PJACW</td>
</tr>
<tr>
<td>____ BLOOD IN EDTA</td>
<td>EDTA</td>
<td>____ OTHER PLASMA</td>
<td>PLMA</td>
</tr>
<tr>
<td>____ EDTA PLASMA</td>
<td>EDTPL</td>
<td>____ RECTAL WASH*</td>
<td>RWASH</td>
</tr>
<tr>
<td>____ FECES</td>
<td>FECES</td>
<td>____ RECTAL WICK (Sno-Strip)*</td>
<td>RWICK</td>
</tr>
<tr>
<td>____ HEPARINIZED BLOOD/CORD</td>
<td>HEBPC</td>
<td>____ RECTAL WICK (Polyfiltronics)*</td>
<td>RPWCK</td>
</tr>
<tr>
<td>____ HEPARINIZED BLOOD/PERIPHERAL</td>
<td>HEBBP</td>
<td>____ SEMINAL PLASMA*</td>
<td>SEMPL</td>
</tr>
<tr>
<td>____ HEPARINIZED PLASMA</td>
<td>HEPPL</td>
<td>____ SEMEN*</td>
<td>SEMEN</td>
</tr>
<tr>
<td>____ INTESTINAL WASH*</td>
<td>IWASH</td>
<td>____ SERUM</td>
<td>SERUM</td>
</tr>
<tr>
<td>____ NASAL WASH</td>
<td>NWASH</td>
<td>____ TEARS</td>
<td>TEARS</td>
</tr>
<tr>
<td>____ PAROTID SALIVA</td>
<td>PAROT</td>
<td>____ VAGINAL WASH*</td>
<td>VWSH</td>
</tr>
<tr>
<td>____ WHOLE SALIVA</td>
<td>WSVL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicates additional information required (see below)

NOTE: Information in shaded areas needs to be entered into the DIRECT SHIPMENT DATABASE

MALE GENITAL TRACT SECRETIONS

Does the volunteer have any signs/symptoms of infection (e.g., abnormal penile discharge, burning with urination?) ______ (1-No, 2-Yes)

Has the volunteer ejaculated within the past 48 hours? ______ (1-No, 2-Yes)

Did the semen specimen fail to liquefy? ______ (1-No, 2-Yes)

If YES is the answer to any of the above questions, the specimen is NOT ACCEPTABLE - DO NOT SHIP SPECIMEN TO MUCOSAL IMMUNOLOGY LABORATORY!

SPECIMEN TYPE: Circle appropriate one(s)

SEmen SEMINAL PLASMA PRE-EJACULATE WICKED

For specimens collected at home/location other than VEU, provide

LENGTH OF TIME FROM COLLECTION TO VEU RECEIPT _______ Hours _______ Minutes _______

RECTAL SECRETIONS (collected by Polyfiltronics wicks)

Is the specimen contaminated with blood? ______ (1-No, 2-Yes)

If YES is the answer to the above question, the specimen is NOT ACCEPTABLE - DO NOT SHIP SPECIMEN TO MUCOSAL IMMUNOLOGY LABORATORY!

FECAL CONTAMINATION ______ (1-No, 2-Yes)
VOLUNTEER ID NUMBER: [_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____][_____]

FEMALE GENITAL TRACT SECRETIONS
Is the volunteer free of abnormal vaginal discharge? [ ] (1-No, 2-Yes)
Has the volunteer had sexual intercourse with vaginal deposit of ejaculate within the past 72 hours? [ ] (1-No, 2-Yes)
Has the volunteer douched within the past 72 hours? [ ] (1-No, 2-Yes)
Is the specimen contaminated with blood? [ ] (1-No, 2-Yes)

If YES is the answer to any of the above questions, the specimen is NOT ACCEPTABLE - DO NOT SHIP SPECIMEN TO MUCOSAL IMMUNOLOGY LABORATORY!

Birth control pill type: Specify ________________________ [ ] None
Does the volunteer have a regular menstrual cycle? [ ] (1-No, 2-Yes)

If NO, circle ALL reasons below that apply

HYSTERECTOMY
OOPHORECTOMY
OTHER (Specify ________________________)

Was the mid-cycle peak determined by urinary LH kit? [ ] (1-No, 2-Yes)

SPECIMEN TYPE: Circle appropriate one(s)

CERVICAL ASPIRATE  CERVICAL WICK  VAGINAL WASH

DAY OF CYCLE [___] post-LMP

INTESTINAL SECRETIONS

METHOD OF SPECIMEN INDUCTION [ ] (1-Ingestion of Golytely, 2-Enema of Golytely)

COLOSTRUM

LENGTH OF TIME COLLECTED POST PARTUM Hours [___] Minutes [___]

SUBMIT THIS FORM WITH THE SPECIMEN DIRECTLY TO THE MIL
APPENDIX D

DISCONTINUED COLLECTION METHODS

COLLECTION OF CERVICAL SECRETIONS BY SNO-STRIPS  [Label = CRSWK]
[Note: Because frequent contamination with blood proteins and low volume of extractable fluid, this should not be considered a method of first choice.]

Supplies:
- 10" Dressing forceps or hemostat
- Sno-Strips
- Lubricating jelly
- Dulbecco's PBS containing 0.01% sodium azide, 0.5 mL
- Sterile vaginal speculum
- Cryovial, 1.5 mL
- Cotton swab

1) Moisten speculum with lubricating jelly. Insert speculum. Remove excess mucus from cervix with a moistened cotton swab.

2) Grasp two stacked ophthalmic tear flow indicator strips (“Sno-Strips”) with 10-in. forceps or a hemostat. Apply the strips to the cervical os until secretions wick up to the “shoulder” of the strip (approximately 10 sec.).

3) Trim the two strips at the shoulder and place the saturated stubs in 0.5 mL Dulbecco's PBS with 0.01% sodium azide.

4) Use two more stacked tear flow strips to collect secretions. Place trimmed strips in the same cryovial as the first two strips so that all four strips are together.

5) Freeze vials at $-20^\circ C$ or $-70^\circ C$ until tested.


COLLECTION OF PRE-EJACULATORY FLUID BY SWAB  [Label = PJACS]

Supplies:
- S/P Culturette swabs
- Sterile collection cup
- Dulbecco’s phosphate-buffered saline (PBS)
- Bovine serum albumin
- Centrifuge tube
- Table-top centrifuge

1) When pre-ejaculate fluid appears, swab the tip of the penis gently with a swab pre-moistened with the vial/gel system provided with the kit to collect droplets. May be repeated as new droplets appear.

2) Ejaculate into the specimen container and close lid.

3) Place the swab containing pre-ejaculatory fluid into the 1 mL vial provided, which contains a saline solution, and cut or break off the swab so that the lid can be closed. (The vial is snapped open to release the saline.)
4) Deliver both pre-ejaculate and semen specimens to the laboratory as soon as possible, preferably within 1 hr, for processing.

5) Process the pre-ejaculatory fluid. In the lab, add PBS containing 1% BSA (700 µL) directly to the tube; vortex for 1 min, then centrifuge at 2,000 x g for 1 min. Remove the swab to another microfuge tube; add 200 µL PBS/BSA, vortex, and centrifuge to elute the Ig remaining in the swab. Combine eluates from both swab-wash steps (volume approximately 1 mL). Immediately prepare a minimum of five aliquots (0.1 - 0.2 mL) into cryovials (0.5 mL capacity) and store at −70°C.


**COLLECTION OF RECTAL SECRETIONS BY SNO-STRIPS** [Label = RWICK]

**Supplies:**
- 10” Dressing forceps
- Anoscope
- Sno-Strips (62x6 mm; 5 mm at the rounded collection tip)
- Surgilube (sterile chlorhexidine gluconate)
- Dulbecco’s PBS containing 0.01% sodium azide, 0.5 mL

1) Lubricate the anoscope with a small amount of Surgilube. With patient bending over the exam table (preferred position by men) or supine in stirrups (preferred position by women), insert the anoscope, cleanse the rectal mucosa with a moist (not wet) cotton applicator. Repeat cleansing step with a second applicator and blot the area to dry.

2) Place two stacked Sno-Strips in a mucosal fold and hold until saturated to the shoulder (around 10 sec). Carefully withdraw and place them on a non-absorbent surface (such as wax paper). Repeat with a second pair of 2 strips. Trim each strip at the shoulder.

   IF BLEEDING OCCURS, withdraw the strips, discard them, and try another site with fresh strips. If blood contamination is not avoidable, terminate the sampling.

3) Place the sample ends of all four trimmed strips into transport medium in a cryovial (1.5 mL capacity). Tap the vial on the table to get the papers down into the solution. Seal tightly; freeze if possible, or refrigerate at 4°C for a few hours, if not possible to freeze sample immediately. Freeze vials as soon after specimen collection as possible at -70°C. Specimens may be batched and sent together frozen on dry ice.

   IF FECAL CONTAMINATION is unavoidable; for example, if adequate cleansing will result in bleeding, sample anyway. Stool can be solubilized in the lab (tiny amounts anyway), whereas a sample with blood cannot be salvaged. We prefer neither contaminant, but stool is better than blood.