WOMEN'S INTERAGENCY HIV STUDY

SECTION 10a: DISCONTINUED LABORATORY SPECIMEN COLLECTION AND PROCESSING PROCEDURES

I. OVERVIEW OF SPECIMEN COLLECTION AND IDENTIFICATION

A variety of specimens were obtained at different visits from each participant enrolled in the WIHS. Specimens were collected by phlebotomy and during the Medical Exam. This section details specimen processing and testing that have been discontinued and are not performed at the current WIHS core visit. In many cases though, these specimens are still available in local and central repositories. See Section C for the Specimens Chart with details on discontinued collection, processing and storage of specimens. These lists include the test name, type of test and, where applicable, specimen storage and shipping information. Appendix B of Section 10 indicates specific tests that were performed at each visit.

A. DESIGNATION OF LAB TESTS AND SPECIMENS

Of the laboratory specimens obtained, some were processed immediately and others were stored for future testing. Where tests are performed varies 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 laboratory, e.g., CBC & Diff.
2. *Central Immediate* - ship overnight to a central lab for processing.
3. *Local Save & Batch* - process and prepare for local storage (as part of a local repository).
4. *Central Save & Batch* - process and prepare for *temporary* storage locally. Some central save and batch specimens will be shipped to a central laboratory every one to three months for testing; others will be shipped periodically the national repository.
5. *Exam Site* - process and prepare immediately for testing in the clinic. Results are recorded on the physical exam form, e.g., KOH and saline preps.

B. IDENTIFICATION OF SPECIMENS

Local WIHS laboratories have been responsible for the labeling and tracking of specimens. Specimens were individually labeled with the Participant ID, specimen date, alpha and/or numeric specimen codes and visit number. Three-month VRS visits were represented as "VV.1" where VV was the standard WIHS core visit number. For example, specimens collected during the VRS visit that occurs three months after WIHS core visit 13 should be labeled "13.1." VRS specimens collected at WIHS core visits should be labeled with regular WIHS visit numbers. WIHS ID's for new recruits will be distinguishable from those of original recruits in that the second digit for all new recruits will be a "2."

Sites were instruction to attach labels to specimens at the time of collection and *prior* to transportation to the lab. Additionally, polyester protective tape (liquid nitrogen safe) may have been placed over the labels and wrapped securely around the tubes before freezing.

C. SPECIMENS CHART

1. BLOOD:

Specimens for Hepatitis Serology, Toxoplasma Serology, HTLV 1& 2, and Herpes Serologies were collected at the baseline visits. RPR Syphilis Screening was performed at visits 4 and 6 for the 1994/95 cohort and at the baseline visit for the 2001/02 cohort. Specimens for insulin, lipid panel, A1C, and glucose have been collected since visit 13. These specimens were sent to the central repository and tested later for fasting participants from visits 13-19. Beginning with visit 20, these specimens were sent for real time testing on all participants, regardless of fasting status. Collection of the lavender for A1C was discontinued at visits 24 - 32. Beginning with visit 24, the tubes for TC, HDL, TRIG, LDL, insulin and glucose were collected at odd visits only. The Cardiovascular Sub study discontinued collection of the SST for novel assays and lavender for NMR Lipoprotein at visit 24.

2. ORAL PROTOCOL SPECIMENS:

The Oral Protocol began at visit 9 and was discontinued after visit 20.

TEST	COLLECTION AND PRIORITY	VOLUME	LAB TYPE	PROCESSING	STORAGE	SHIPMENT	COMMENTS
Stimulated Saliva Evaluation	Collect stimulated saliva into a graduated test tube	Record volume to the nearest 1/4 ml	Freeze locally; later to be shipped to central lab. If randomly selected, an extra 2 ml aliquot is sent to central lab for immediate testing	Vortex and aliquot into cryotubes and freeze immediately at - 70°(C)	- 70°(C)	Batch ship centrally to repository. If randomly selected, overnight ship extra 2 ml aliquot with polar packs to Dr. Jorgen Slots	
Subgingival plaque	Paper points in glass vial with transport medium and paper points in plastic vial for PCR analysis	N/A	Central Immediate	N/A	None	Overnight mail to Dr. Jorgen Slots at room temperature	Shipping materials provided by Dr. Slots
Erythematous candidiasis	Wooden spatula	N/A	Central Immediate	Wet smeared slides with fixative, let dry	None	Ship to Oral Pathology Laboratory	Smear kits provided by Dr. Joan Phelan

3. URINE SPECIMENS:

TEST	COLLECTION AND PRIORITY	VOLUME	LAB TYPE	PROCESSING	STORAGE	SHIPMENT	COMMENTS
Urine for Chlamydia/ Repository	Beginning stream in Sterile Container	10 ml	Central Save & Batch	Vortex 10 ml sample, place entire specimen in either 5 x 2 ml or 10 x 1 ml aliquots	- 70°(C)	To repository on dry ice from visits 1 through 19	

4. VAGINAL SPECIMENS:

There are no vaginal specimens that have been discontinued at this time.

5. CERVICAL SPECIMENS:

TEST	COLLECTION AND PRIORITY	VOLUME	LAB TYPE	PROCESSING	STORAGE	SHIPMENT	COMMENTS
Syphilis	Dacron-tipped swab	N/A	Central Save and Batch	Allow slide to air dry, then circle perimeter of specimen on slide	Store at room temperature until shipped	Batch ship at room temperature to CDC	Instructions for shipping in Section V

6. CERVICAL-VAGINAL LAVAGE:

There are no cervical-vaginal lavage specimens that have been discontinued at this time. Participants included in the NIDA substudy between visits 6 and 14 had 4 vials of CVL redirected towards that substudy during those visits.

7. HHV-8 PROTOCOL SPECIMENS:

TEST	COLLECTION AND PRIORITY	VOLUME	LAB TYPE	PROCESSING	STORAGE	SHIPMENT	COMMENTS
Anal specimens	HHV-8 positive participants only; Dacron swab	N/A	Central Save and Batch	Return swab to sterile dry sleeve collector	Store at -70° C, if possible, otherwise -20° C acceptable	Batch ship to repository on dry ice	
Stimulated Saliva	HHV-8 positive participants only; collect saliva into a graduated test tube	Record to nearest 1/4 ml		Pipet and aliquot into vials and freeze immediately at -70° C	Store at -70° C		
Serum, Plasma and Cells for Repository	San Francisco only; HHV-8 positive participants only	Protocol for collection of serum, plasma and cells to be distributed only at San Francisco WIHS site.					Phlebotomy to be done only at the San Francisco site

8. OTHER CLINICAL EXAMS:

Tuberculin PPD testing was discontinued after visit 16. The Mantoux Skin Test Result Anergy Panel was discontinued after visit 11.

II. BLOOD SPECIMENS

A. PHLEBOTOMY VOLUMES AND PRIORITIES FOR THE ORDER OF BLOOD DRAW AT THE BASELINE VISIT FOR THE 1994/95 COHORT

Tube Priority	Tube Color	Tube Volume	Test	Notes
1	Red-Top	1-2 ml	HIV Ab	
2	Purple-Top	2-5 ml	CBC & Diff	Priority 2 & 3 may be done on the same tube.
3	Purple-Top	2-5 ml	T-Cell Subsets	Priority 2 & 3 may be done on the same tube.
4	CPT	8 ml	Repository	
5	CPT	8 ml	Repository	
6	Red-Top	10 ml	Save & Batch Serology	
7	Red-Top	2-5 ml	Liver & Renal Function	
8	Red-Top	2 ml	Hepatitis B&C	
9	Red-Top	2-5 ml	Syphilis	
10	Red-Top	10 ml	Serum	
11	CPT	8 ml	Repository	
12	Red-Top	10 ml	Repository	
13	CPT	8 ml	Repository	

Totals Baseline Visit for Original Recruits:
Red-top tube = 37 ml – 44 ml
Purple-top tube = 4 ml – 10 ml
CPT = 32 ml

B. PHLEBOTOMY VOLUMES AND PRIORITIES FOR THE ORDER OF BLOOD DRAW AT THE BASELINE VISIT FOR THE 2001/02 COHORT

Tube Priority	Tube Color	Tube Volume	Test	Notes
1	Red-Top or Tiger-Top SST	1-2 ml	HIV Ab	Not required on HIV-positive women with hard-copy documentation of positive Western blot result.
2	Purple-Top	2-5 ml	CBC & Diff	Priority 2 & 3 may be done on the same tube
3	Purple-Top	2-5 ml	T-Cell Subsets	Priority 2 & 3 may be done on the same tube.
4	CPT	8 ml	Repository	Includes plasma to be used for viral quantification by RNA PCR.
5, 6, 7, 8	CPT	8 ml each	Repository	
9	Red-Top or Tiger-Top SST	4 ml	Save and batch serology	Testing for HTLV 1 & 2 and HSV. Will be batch shipped to repository with redirect to appropriate labs.
10	Red-Top or Tiger-Top SST	2-5 ml	Liver/Renal Function	
11	Red-Top or Tiger-Top SST	2 ml	Hepatitis B & C	
12	Red-Top or Tiger-Top SST	2 ml	RPR Syphilis	
13	Tiger-Top SST	5 ml	Lipid Panel/Insulin	
14	Purple-Top (pediatric)	2.5 ml	Hemoglobin A1c	
15	Gray-Top (3 ml size)	3 ml	Glucose	
16	Red-Top or Tiger-Top SST	10 ml	Serum	
17	Red-Top or Tiger-Top SST	10 ml	Repository	

Totals Baseline visit for New Recruits:
Red-top tube or Tiger-top SST = 31 ml – 35 ml Tiger-top SST = 5 ml
Purple-top tube = 6.5 ml – 12.5 ml Gray-top tube = 3 ml
CPT= 40 ml

C. PHLEBOTOMY VOLUMES AND PRIORITIES FOR THE ORDER OF BLOOD DRAW AT VISIT 2 FOR THE 1994/95 COHORT

Tube Priority	Tube Color	Tube Volume	Test	Notes
1	Red-Top	1-2 ml	HIV Ab	Not required after visit one on HIV-positive women.
2	Purple-Top	2-5 ml	CBC & Diff	Priority 2 & 3 may be done on the same tube.
3	Purple-Top	2-5 ml	T-Cell Subsets	Priority 2 & 3 may be done on the same tube.
4	CPT	8 ml	Repository	Includes plasma to be used for viral quantification by RNA PCR.
5	CPT	8 ml	Repository	
6	CPT	8 ml	Repository	
7	CPT	8 ml	Repository	
8	CPT	8 ml	Repository	
9	Red-Top	10 ml	Repository	

Totals Visit 2:
Red-top tube = 11 ml – 12 ml, Purple-top tube = 4 ml – 10 ml, CPT = 40 ml

D. PHLEBOTOMY VOLUMES AND PRIORITIES FOR THE ORDER OF BLOOD DRAW AT ALL FOLLOW-UP VISITS THROUGH VISIT 19

Tube Priority	Tube Color	Tube Volume	Test	Notes
1	Red-Top or Tiger-Top SST	1-2 ml	HIV Ab	Not required after visit one on HIV-positive women.
2	Purple-Top	2-5 ml	CBC & Diff	Priority 2 & 3 may be done on the same tube. For HIV-negative women, required only at even-numbered visits beginning with visit 11.
3	Purple-Top	2-5 ml	T-Cell Subsets	Priority 2 & 3 may be done on the same tube. For HIV-negative women, required only at even-numbered visits beginning with visit 11.
4	CPT Tube	8 ml	Repository	Includes plasma to be used for viral quantification by RNA PCR.
5, 6, 7, 8	CPT Tube	8 ml each	Repository	
9	Red-Top or Tiger-Top SST	2-5 ml	Liver/Renal Function	Prior to visit 14, collected annually at odd-numbered visits. Beginning with visit 14, collected at all visits.
9	Red-Top or Tiger-Top SST	2-5 ml	RPR Syphilis	Collected at Visits 4 and 6.
10	Tiger-Top SST	5 ml	Lipid Panel/Insulin	Collected on all participants beginning at visit 13. Collected once per year only beginning at visit 24.
11	Purple-Top (pediatric)	2.5 ml	Hemoglobin A1c	Collected on all participants at visits 13-23.
12	Gray-Top (3 ml size)	3 ml	Glucose	Collected on all participants beginning at visit 13. Collected once per year only beginning at visit 24.
13	Red-Top or Tiger-Top SST	10 ml	Repository	

Totals After Visit 2:
Red-top tube or Tiger-top SST = 12 ml – 17 ml
Purple-top tube = 2.5 ml – 12.5 ml
CPT = 40 ml
Tiger-top SST = 5 ml
Gray-top tube = 3 ml

E. BLOOD SPECIMEN DESIGNATIONS

1. Discontinued tests slated for local immediate processing are as follows:

   a. Syphilis Screening (2-5 ml serum) at baseline and at Visit 4 and Visit 6
   b. Hepatitis B & C (2-5 ml serum) at baseline

2. Discontinued samples slated for Local and Central (Repository) Save & Batch:

   a. Plasma and Cell Repository

      Baseline: Original Recruits
      Process 32 ml of whole blood from CPTs during the baseline visit. Plasma and cells should be aliquoted as follows: 10.0 ml plasma for central and 6.0 ml plasma for local repositories. From same CPT above: 22 million cells for central and 18 million cells for local repositories.

      Baseline: New Recruits
      Process 40 ml of whole blood from CPTs during visit 2. Plasma and cells should be aliquoted as follows: 10.0–13.0 ml plasma for central and 6.0–7.0 ml plasma for local

repositories. From same CPT above: 22–26 million cells for central and 18–22 million cells for local repositories.

Follow-up Visits
Processing of viable cell pellets for the central repository was discontinued at the start of visit 27. Plasma aliquots at 0.5ml were discontinued at the start of visit 27.

b. Serum Repository

Baseline: Original Recruits and New Recruits
Process 20 ml whole blood from red-top tube and aliquot serum as follows: 7 ml for central and 3 ml for local repositories.

c. Additional Serum Specimens

At Baseline (Original recruits) only:
An additional 5 ml will be frozen and stored locally for batch shipping to central labs (central save and batch) as follows:

- Toxoplasmosis (2 x 0.5 ml tubes serum), stored in separate boxes, send one (save second as back-up) to Jack Remington's lab.
- HTLV 1&2 (2 x 0.5 ml tubes serum), stored in separate boxes, send one (save second as back-up) to Dr. William Hardy's lab.
- HSV Serology, B-2 Microglobulin, Neopterin (3 ml serum).

All serum and cell pellets are to be frozen at -70° C. All plasma and other cells are to be stored in vapor-phase liquid nitrogen or -150° C freezers.

At Baseline (New recruits) only:
An additional 2 ml will be frozen and stored locally for batch shipping to central labs (central save and batch) as follows:

- HTLV 1&2 (2 x 0.5 ml tubes serum), stored in separate boxes, send one (save second as back-up; store locally) to repository for redirect to Dr. William Hardy's lab.
- HSV Serology (2 x 0.5 ml tubes serum) shipped together, as pairs, to repository for redirect to Dr. Corey's lab. Sera must be shipped in divided boxes.

All serum and cell pellets were frozen at -70° C. All plasma and other cells were to be stored in vapor-phase liquid nitrogen or -150° C freezers. Plasma was approved for storage at -70° C beginning with visit 17.

F. SERUM CREATININE TESTING HISTORY

In 2007, sites were queried about serum creatinine testing specifics:

1. *Bronx and Washington, DC consortia*: Creatinine was performed by the Baltimore Quest Diagnostics facility. Direct Alkaline Picrate from Olympus America has been in use at Quest since January 1995. The method was run on the Olympus AU5200 analyzer from January 1995 to May 2004. It has been run on the Olympus AU5400 since June 2004.

   Current, and typical, assay precision - between-run:

Level	N	mean, mg/dL	CV (%)
Low	23,696	0.92	4.38
Mid	27,106	2.50	2.59
High	26,910	9.79	2.16

2. *Brooklyn consortium*: Before November 2005 the laboratory used Vitros 900 to measure creatinine. The method was based on adding Picric Acid to the sample. Since November 2005 the instruments used are Olympus AU640 and AU2700. The reagent used contains Picric Acid which reacts with creatinine and forms a yellow-orange complex. The rate of change in absorbance at 520/800 nm is proportional to the creatinine concentration in the sample.

3. *Los Angeles consortium*: The lab uses Coulter CX5 instruments. The method is the modified rate Jeffe method. In the reaction, creatinine combines with picrate in an alkaline solution to form a creatinine-picrate complex. The change of absorbance at 520 nanometers is directly proportional to the concentration of creatinine.

4. *San Francisco consortium*: The lab uses the Olympus creatinine procedure. The method uses the AU500 and AU5200 analyzers and is a kinetic modification of the Jaffe procedure. Estimates of precision, based on NCCLS recommendations are consistent with typical performance.

AU500

N=50	Within Run		Total	
	SD	CV%	SD	CV%
1.6 mg/dL	0.031	1.98	0.042	2.64
7.5 mg/dL	0.094	1.26	0.158	2.10

AU5200

N=50	Within Run		Total	
	SD	CV%	SD	CV%
1.6 mg/dL	0.046	2.60	0.050	2.90
5.5 mg/dL	0.040	0.64	0.040	0.64

5. *Chicago consortium*: Chicago WIHS has used Quest Diagnostics in Wood Dale, IL since visit #1 (1994-2007). The assay, or method, is the enzymatic method performed on the Olympus 5400 SP (spectrophotometry) for serum creatinine. The test to test variability, or precision, at their lab is 15%, or +/- 0.3mg/dl; this figure may have changed since 1995 (visit #1).

G. LABORATORY DESIGNATIONS BY TEST

Exam Site	Local Immediate	Central Immediate	Local Save & Batch	Central Save & Batch
N/A	• Hepatitis B & C* • RPR Syphilis screening test**	N/A	• 6 ml plasma repository at baseline • 18 million cells repository at baseline • 3 ml serum repository at baseline	• Toxoplasmosis* • HSV Serology* • HTLV 1 + 2* • Serum repository 7 ml at baseline • Cells repository 22 million at baseline • Plasma repository 10 ml at baseline

* Baseline visit only (both original and new recruits)

** Baseline visit (both original and new recruits), visit 4 and visit 6

H. PROCESSING PLASMA FROM CELL PREPARATION TUBES

Baseline Visit (Original Recruits):

i. 6 x 0.5 ml aliquots (central repository)
ii. 7 x 1.0 ml aliquots (central repository)
iii. Remainder (local repository) site-specific aliquot volumes

Baseline Visit (New Recruits):

i. 6-7 x 0.5 ml aliquots (central repository)
ii. 7-9 x 1.0 ml aliquots (central repository)
iii. Remainder (local repository) site-specific aliquot volumes

I. PROCESSING SERUM FROM RED-TOP TUBES

Baseline Visit (Original Recruits):

- 4 X 0.5 ml and 5 X 1.0 ml aliquots in 1.8 ml labeled cryotubes (for central repository). The laboratory technician will need to complete the participant ID, date and visit number the specimen was drawn plus the S-code on all labels.
- 10 X 0.5 ml aliquots for save and batch serologies: Toxoplasmosis, HSV, B_2 microglobulin, Neopterin, HTLV 1 + 2 (i.e., total 1 ml per test: aliquoted in 0.5 ml/tube).
- 1.8 ml cryotube labeled with the log#, "S" (serum) and date (for local repository).

Baseline Visit (New Recruits):

- 4 X 0.5 ml and 5 X 1.0 ml aliquots in 1.8 ml labeled cryotubes (for central repository). The laboratory technician will need to complete the participant ID, date and visit number the specimen was drawn plus the S-code on all labels.
- 4 X 0.5 ml aliquots for save and batch serologies: HSV, HTLV 1 + 2 (i.e., total 1 ml per test: aliquoted in 0.5 ml/tube).
- 1.8 ml cryotube labeled with the log#, "S" (serum) and date (for local repository).

J. PROCESSING WHOLE BLOOD FROM LAVENDER TUBES (for A1C testing)

A1C testing from Whole Blood collected in Lavender tubes was performed from visits 13-19 on banked specimens. These specimens were sent to Quest Diagnostics for immediate testing from visits 20-23. Collection of the lavender for A1C was discontinued at visits 24 - 32.

1. Blood should be collected aseptically in a Lavender tube.
2. Gently invert tube approximately five times immediately after filling to make sure the anticoagulant and blood are well mixed.
3. If tubes are shipped to a processing lab, they should be shipped on -20° C cold packs when the ambient temperature is above 75° F as described above in J.5.
4. Processing labs should invert tubes five times immediately prior to aliquoting. Aliquot serum in 0.5 amounts to 1.8 ml labeled cryotubes. Label all specimens according to protocol and ensure correct fasting vs. non-fasting specimen codes.
5. Freeze at -70° C.

III. COLLECTION AND PROCESSING OF ORAL SPECIMENS OBTAINED DURING PHYSICAL EXAM

A. CANDIDA SWAB ON CULTURETTE (BASELINE ONLY – ORIGINAL AND NEW RECRUITS)

- Rotate a sterile, rayon-tipped applicator (Culturette) on both sides of the buccal mucosa or any site with a lesion.
- Reinsert the swab into the culturette tube and break the tube's ampule to release the storage media.
- Affix the study ID label, preprinted with participant's ID. Record the date, S-code and visit number, and an "O" on the label, to identify the specimen site as oral.
- Immediately store the culturette in the refrigerator and quickly (less than eight hours) transport to the lab (on ice if possible).
- Shafts are to be broken off and swabs placed in protocol specified glycerol media (see Appendix I of WIHS Laboratory Manual) in a 1.8 ml cryotube, stored at -70° C and shipped to repository.

NOTE: Oral Candida cultures will be done at baseline only (both original and new recruits).

B. HSV CULTURE OF ORAL ULCERS OR FISSURES (BASELINE ONLY –ORIGINAL AND NEW RECRUITS)

- Using a sterile, dacron-tipped swab, obtain the material from the base of ulcers and fissures. Follow your local lab procedures for processing of the HSV culture. HSV typing will be done for HSV Type HSV Type II and I. Please note that Varicella Zoster Virus (VZV) typing is not required by protocol as part of Herpes testing. Please complete your local lab requisitions so that your lab limits typing to those two types. However, Question A6 on Form L17 will allow recording of VZV should it be reported by your local laboratory. In addition, if the culture is Herpes positive but not typed, sites should record "positive, not typed" on the Herpes Culture lab form, Form L17.

NOTE: Oral HSV cultures will be done at baseline only (both original and new recruits).

C. TEST DESIGNATIONS

Exam Site	Local Immediate	Central Immediate	Local Save & Batch	Central Save & Batch
	• HSV culture oral ulcers & fissures (baseline only*)			• Culture for oral Candida (baseline only*)

* Both original and new recruits

D. EXPANDED CVL PROCESSING PROTOCOL

This Expanded CVL Processing Protocol was optional for WIHS sites that are able to process the CVL specimens according to the guidelines outlined below. All sites discontinued this protocol by visit 27.

All CVL specimens will be processed rapidly (within six hours). A cell count will be prepared from the cell pellet at sites that possess the necessary equipment or technical support; it is optional for sites that do not possess the necessary equipment or technical support. Rapid processing by the local laboratory is essential to insure the stability of the RNA. Immediate sample processing (within six hours) is required. Note time sample was obtained, time of processing and time of freezing on the WIHS CVL Processing Form.

Cervicovaginal Lavage: Local Laboratory Procedures to Separate Cellular and Supernatant Components

a. Supplies for Local Laboratory Procedures:

- 15 cc conical tubes
- 1.5 cc cryovials
- 10 cc sterile syringe
- Chemstrip-OB (Boehringer Mannheim Diagnostics, Cat #417144) for VCS guaiac and leukocyte testing
- Sterile saline

b. Preparation of CVL Specimen by Local Laboratory

i. Note the volume, color, and presence of gross blood by visible inspection.

ii. Gently invert the specimen to thoroughly mix the cellular and fluid components.

iii. Remove 6.0 ml CVL from total volume obtained.

a) Aliquot as follows:

- 3 ml (3 x 1 ml aliquots) for HPV Testing and freeze at -80° C.
- 2.5 ml (2 x 1 ml and 1 x 0.5 ml) for HIV RNA PCR (cells and supernatant) and freeze at -80° C.
- 0.5 ml for quality assurance tests (Chemstrip Tests).

b) On the 0.5 ml aliquot designated for quality assurance tests, check for presence of blood and leukocytes using the ChemStrip-OB indicator strip. Complete Chemstrip OB tests according to the directions provided in the package insert.

iv. Centrifuge the remaining 3-4 ml of CVL specimen at 600 x g for 15 minutes into cells and supernatant.

c. Supernatant

i. Carefully pour supernatant off the cell pellet. Reserve cell pellet for further processing (see below).

ii. Note total volume of supernatant

iii. Supernatant should be divided into 2 x 1 ml aliquots and 2-4 x 0.5 ml aliquots, each of which should be placed in a 1.5 ml cryovial. Label all cryovials and store at -80° C.

d. Cell Pellets

i. Using graduations on centrifuge tube containing the cell pellet, bring volume up to 2.2 ml with saline and resuspend with gentle mixing or pipetting.

ii. Perform the Round Cell Stain test and cell count on the volume of well-mixed cell suspension indicated in the manufacturer's package insert.

iii. Gently and thoroughly resuspend cells and aliquot remaining cell suspension as 8 x 250 ul (.25 ml) and place in 1.5 ml cryovials.

Label all eight vials and store at -80° C until batch shipment. Labels need to include the specimen codes as follows:

- CVL for the whole CVL specimen
- CVS for the CVL Supernatant
- CVP for the CVL Pellet

e. Shipping

Batch ship supernatant sample and cell pellet sample to central repository.

NOTE: For those using the modified CVL separation protocol, 50% of all available samples MUST be sent to the Central Repository.

f. Round Cell Stain Test: VCS Cell Pellet

PLEASE NOTE: The Round Cell Stain Test from Humagen cat#112-RCS is no longer available. Local laboratories will follow the guidelines according to the protocol outlined below:

Intended Use: For the identification of peroxidase positive cells in cervical vaginal lavage.

Summary: Peroxidase staining is an inexpensive, quick screen for WBCs in CVL. Peroxidase positive cells include neutrophils, eosinophils, and macrophages. Monocytes may stain faintly. Basophils and lymphocytes will not stain.

Expected Result: Cytoplasmic granules in perozidase positive cells will stain brown.

Reagents:

Part 1: 0.001M benzidine

- 95-100% Ethyl alchol(not denatured) 30% final concentration.
- Dihydrochloride benzidene (Sigma #B3383) 2.1 mg/ml final concentration.
- Ultrapure water.

1) Determine total volume to be made. Weigh out the dihdrochloride benzidine needed (2.1 mb x total volume in ml). This chemical is light sensitive and carcinogenic at high concentrations – HANDLE WITH CARE.
2) Dissolve the dihydrochloride benzidine in ethyl alcohol. (30% of the total volume). Do this in a polypropylene tube wrapped in aluminum foil. This takes time to go into solution.
3) Hand shake or vortex for 5 minutes and leave in a dark place at room tempature for 30 minutes.
4) Add ultrapure water to the mixture to bring it to the final volume. This may be slightly brown in color and may have some white precipitate. Store refrigerated away from light.

Part 2: 0.038% peroxide

- 0.005% Hydrogen Peroxide in Phosphate Buffered Saline, pH 7.0
  Phosphate Buffered Saline- 7.6 g NaCl.
- 1.93 g Sodium Phosphate-dibasic.
- 0.36 g Sodium Phosphate-monobasic.
- Bring up to 50 ml with ultrapure water.

1) Check pH. Adjust up to 7.0 by adding monobasic sodium phosphate, adjust down to 7.0 using dibasic sodium phosphate. This can be stored up to 1 year. Determine total volume PBS needed.

2) Add hydrogen peroxide to a final concentration of 0.05%.

3) Store refrigerated at 4C.

Procedure:

1. In a clean vial, mix 10uL each of benzidene solution (Part 1) and peroxide solution (part 2).
2. Add 10uL of CVL pellet solution from final stage of expanded CVL processing protocol.
3. Mix well and incubate at room temperature (20-25C) for 5-10 minutes.
4. Load Neubauer hemacytometer chamber. Let sit at least 10 seconds.
5. Count stained and unstained round cells in 5 large squares. Record results on Form L19.

Troubleshooting: If stain becomes faint, both benzidine and peroxide solutions should be replaced. Each solution is stable when refrigerated at 4C for one year.

Reference: Humagen, Fertility Diagnostics, Inc. Product insert RCS 1095500.

IV. COLLECTION AND PROCESSING OF URINE SPECIMENS

At the baseline visit (both original and new recruit) and odd annual visits (i.e., 1, 3, 5, etc.) through visit 19 sites collected a first stream urine sample [10 ml] for LCR (Chlamydia) testing and repository. At the baseline visit for new recruits, an additional 20 ml of first stream urine was collected for local, real time GC and Chlamydia LCR testing. The LCR was **not** obtained as part of the CVS. Specimens were to be collected in a sterile cup with a screw top.

A. CHLAMYDIA LCR SAMPLE (repository) – ODD VISITS (1, 3, 5, etc.) ONLY AND AT BASELINE VISITS FOR NEW RECRUITS

This sample was discontinued for repository use after visit 19.

1. Instruct the participant not to void for one hour prior to sample collection (prior to pelvic exam).
2. Collect 10 ml (beginning stream) in a sterile container and transfer to the lab.
3. In the lab, the urine should be gently vortexed, and the entire specimen should be placed in either five 2 ml aliquots or ten 1 ml aliquots. These should be frozen at -70°C (without being centrifuged) and shipped to repository on dry ice.

B. GC/CHLAMYDIA LCR SAMPLE (LOCAL/REAL TIME) – BASELINE FOR NEW RECRUITS

1. Instruct the participant not to void for one hour prior to sample collection (prior to pelvic exam).
2. Collect 20 ml (beginning stream) in a sterile container.
3. Process according to your local protocol.

C. TEST DESIGNATIONS

Urine tests for WIHS include:

Exam Site	Local Immediate	Central Immediate	Local Save & Batch	Central Save & Batch
• Urine* Pregnancy	• Urine GC & Chlamydia LCR**			• Urine LCR/repository (when applicable)

* A pregnancy test was required **at baseline** for any woman who missed one period (last/most recent period) unless she was:

a. pre-menarche
b. s/p hysterectomy
c. post menopausal
d. currently pregnant

** Baseline visit for new recruits only.

D. URINALYSIS TESTING HISTORY

In 2007, sites were queried about urinalysis testing specifics:

1. *Bronx consortium*: Urinalysis was performed by the Baltimore Quest Diagnostics facility. CLIA-licensed laboratories are required to maintain retired Standard Operating Procedures (SOP) for two years beyond their retirement. Therefore, SOP records of this sort from WIHS Visits 1 through 7 are not available. Quest's current SOP notes:
   - Dipstick - (From at least 2000 onward) Automated Dipstick Analyzer (Clinitek Atlas) using Bayer sticks
   - Microscopic - Manual microscopic evaluation until January 2005
   - Sysmex UF100 automated "microscopic" analysis from January 2005 onward with confirmation by manual microscopy
2. *Brooklyn consortium*: Urinalysis was performed in the clinic using dipsticks.
3. *Washington, DC consortium*: Urinalysis was performed in the clinic on fresh midstream clean catch urine, using the Bayer Reagent Strips (Multistix 10 SG). These Strips are for professional use in near-patient (point-of-care) and centralized laboratory locations. They are ready to use upon removal from the bottle and the entire strip is disposable. The strips may be read visually, requiring no additional laboratory equipment for testing. The Bayer Reagent Strips are CLIA waived when read visually.
4. *Los Angeles consortium*: No reply.
5. *San Francisco consortium*: Urinalysis was performed in the clinic using Bayer chem strips.
6. *Chicago consortium*: Urinalysis was performed in the clinic probably using Bayer Multistix.

V. COLLECTION AND PROCESSING OF GENITAL SPECIMENS OBTAINED DURING PHYSICAL EXAM

A. LESION SWABS AND SLIDES

In this study all ulcerations and fissures will be cultured for HSV **at baseline only (original and new recruits).** DFA specimens will be obtained for syphilis on ulcers and fissures at every visit until the CDC stopped testing these specimens (July 2009). If there is more than one lesion type, i.e., two or more distinctly different appearing lesions, cultures of each should be obtained. In sampling from patients with multiple lesions, keep in mind that vesicle fluid and pustules are the most likely to be herpes simplex virus culture-positive. Fresh ulcers are more likely to yield the virus or to be DFA positive than crusted, healing lesions.

NOTE: External lesions, located on thighs, pubis, perineum or vulva, will be swabbed prior to introduction of talcum powder or lubricant into the vagina.

Swab(s) and slide(s) of vaginal and/or cervical lesions are to be obtained (cervical swab # 3) just prior to performing cervical-vaginal lavage **at the baseline visit (original and new recruits).** Swab(s) and slide(s) of vaginal and/or cervical lesions are to be obtained last, **(after performing the cervical-vaginal lavage, cervical swab #1 and #2 and Pap smear) at follow-up visits.**

Please note that Varicella Zoster Virus (VZV) typing is not required by protocol as part of Herpes testing. However, Question A6 on Form L17 will allow recording of VZV should it be reported by your local laboratory. In addition, if the culture is Herpes positive but not typed, sites should record "positive, not typed" on the Herpes Culture lab form, Form L17.

1. SPECIMEN COLLECTION FOR HSV CULTURE

   After removal of scabs or crusts, ulcers should be rubbed firmly with a sterile dacron swab moistened with viral transport medium. In moist tissues such as the vagina, the swab may be held in place for several seconds and then rubbed against the lesion. Swabs are then placed in the cold viral transport medium before drying occurs. The swabs are vigorously twirled in the medium, pressed against the side of the vial and then discarded. Please note that your local lab procedures should supersede these directions. The viral culture specimens are then sent to a local service laboratory.

   NOTE: Closed vesicles should be broken, and specimen obtained using a sterile swab.

2. SPECIMEN COLLECTION FOR SYPHILIS DFA SLIDE

   Exudate is cleaned off the lesion with a sterile gauze pad. If necessary, compress the base of the lesion or apply suction to the lesion to promote accumulation of serous fluid on the lesion surface. Apply a clean glass slide to promote accumulation of serous fluid on the lesion surface. Apply a second clean glass slide to the oozing lesion or use a sterile bacteriological loop to obtain serous fluid and place it on a glass slide. A sterile syringe (without needle) may be used to aspirate serous fluid from vaginal or cervical lesions. The slide is allowed to air dry. Once the specimen has dried, use a wax pencil to circle the perimeter of the specimen on the slide. Place slides in a plastic transporter box, use tissue or Chux to pad the slide to prevent breakage during transport. Store at room temperature. The slides may be sent at room temperature to the CDC, 1600 Clifton Road, Bldg. 1, Room 3318, Mail stop D-13, Atlanta, GA 30333, Attn: Martha Sears or Dr. Victoria Pope.

B. VAGINAL SWABS

See Section 9 of the Manual of Operations for the order of specimen collection during baseline and follow-up gynecological examinations.

1. Vaginal Swab on Culturette:

Specimens for fungal culture are obtained using a swab from a sterile saline culturette. Rotate the swab on the left and right lateral vaginal walls. The swab is then returned to the culturette, where the saline vial is broken, thus moistening the swab in its plastic tube. Send to local lab for storage and eventual Candida culture, as described in Appendix I in the Laboratory Manual. Record a "V" on the participant ID label to identify the source as vaginal. **Vaginal candida cultures will be done at baseline only (original and new recruits).**

C. CERVICAL SWABS AND PAP SMEAR

1. Cervical Swab for Gen-Probe:

Remove excess mucus from cervical os and surrounding mucosa using one of the swabs provided in the PACE specimen collection kit (Gen-Probe kit). Discard the swab and place the second swab from the collection kit 1–1.5 cm into the endocervical canal. Rotate the swab for 30 seconds; withdraw the swab carefully to avoid contact with the vaginal mucosa. Insert the swab into the Gen-Probe transport tube (which contains a stabilizing fluid). Snap the shaft at the scored line, and cap the tube tightly. The tube may be stored at room temperature or in the refrigerator. **GC & Chlamydia Gen-Probe is to be run at the local lab immediately. Cervical Swab #1 for Gen-Probe is to be collected at Baseline for original recruits only.**

2. Cervical Swab for LCR:

Use the wire shafted swab in the Abbott LCR collection/transport tube. Apply pressure to properly close the tube. Specimens should be immediately refrigerated and transported to the lab. Specimens are to be stored upright in site local repositories, in a -70 C° freezer, for eventual testing. Collect and freeze a cervical swab for LCR testing at every visit. **Cervical Swab #2 for LCR is collected only at Baseline (original recruits only), Visit 1, Visit 2, and Visit 3. This swab is no longer collected starting with Visit 4.**

NOTE: Cervical swab for Gen-Probe and cervical swab for LCR are not obtained from women with no cervix present. In addition, please note that Question A4 on Forms L09 (Chlamydia Gen-Probe) and L13 (Gonorrhea Gen-Probe) asks for the "DATE OF TEST." For sites that have laboratory reports that do not report the "DATE OF TEST," record the report date in this field.

3. Cervical Swab for HIV RNA Quantitation:

From visit 12 through visit 28, and at the 2001/02 recruits baseline visit, a cervical swab for HIV RNA quantitation will be collected prior to collection of CVL.

A. COLLECTION

1. The swabs for HIV RNA quantitation collections must be made from 100% synthetic materials. Puritan Sterile Dacron Polyester Tip Applicator 25-806-1PD is recommended.
2. HIV RNA viral transport media: activated 4M guanidine isothiocyanate solution, GUSCN – 50 ml of 4 M guanidine isothiocyanate (GIBCO cat# 15577-018) with 350 µl of mercaptoethanol (SIGMA cat#M-6250). This solution is stable for 30 days. The transport media is the same as has been provided to sites for DATRI 009 and is described in the

DATRI 009 protocol Section 4.2.1. The local processing lab will provide vials with 1 ml of transport medium. Prior to collection of the specimen, vials should be refrigerated at 4°C.

3. Using a Dacron-tipped swab with plastic shaft, gently insert into the vagina until it reaches the cervix. Rotate the swab at least 360 degrees on the os, and if possible, rotate 720 degrees. In moist tissues such as the vagina, the swabs may be held in place for several seconds. Perform this procedure BEFORE collecting CVL. A smaller swab may also be used if a woman's cervical anatomy or personal comfort level warrants it (Harwood Products Co., order number 25-800D or 25-801D). If the participant has had a hysterectomy, obtain the specimen from the vaginal cuff.
4. Dacron tip must be placed in the appropriate vials before drying occurs and then sealed.
5. Fix the appropriate label to the transport vial and store until shipping. If the specimen was obtained from the vaginal cuff instead of the cervix, label the specimen as "vaginal," not "cervical." HIV PCR swabs can be stored at 4°C for up to 72 hours. After that, vials should be stored at 70°C until testing. Ship on dry ice with regular batch shipments of WIHS core specimens to the Central Repository where they will be stored until testing.

B. PROCESSING

1. HIV PCR swabs can be stored at 4°C for up to 72 hours. After that, vials should be stored at 70°C until testing.
2. Before testing for HIV RNA, remove vial from freezer, thaw and vigorously shake for 30 seconds and then vortex (high setting) for an additional 30 seconds. Remove and discard Dacron tip. Use the whole volume of transport medium for testing.

D. ANAL SPECIMEN COLLECTION

Anal specimens will be collected from HHV-8 positive participants at WIHS core visits 10, 11 and 12. Brush one dacron swab over the anorectal area and return to a sterile dry sleeve collector. It is acceptable to use a Dacroswab similar to those used for viral culture and break it off into a sterile, screw cap, plastic 1.5–2.0 ml tube. Transport the swab at room temperature to the local lab and store at -70° C. It is acceptable to store at -20° C at the local collection site; however, the sample should be archived at -70° C prior to shipment, if possible. Ship to repository on dry ice with regular batch shipments of WIHS core specimens.

ANAL SUBSTUDY SITES ONLY: Sites involved in Joel Palefsky's anal HPV substudy will also need to collect one additional Dacron swab from participants enrolled in the substudy.

Baseline Visit (both original and new recruits): Vaginal and Cervical:

Exam Site	Local Immediate	Central Immediate	Local Save & Batch	Central Save & Batch
• T Vaginalis (Wet Mount) • KOH Prep • pH • Amine (odor) test • T. Vaginalis Culture (Optional)	• HSV Culture (Lesions) • GC/Chla-mydia Gen-Probe (original recruits only)	• Pap Smear	• Chlamydia (LCR swab) (original recruits only) • CVL Fluid	• Culture for Candida • B.V. Slide • Syphilis DFA Slide • CVL Fluid • Anal specimens* • Cervical swab #4 – new recruits only

* New recruits: HHV-8 positive or anal substudy participants only.

Follow-Up Visits: Vaginal and Cervical:

Exam Site	Local Immediate	Central Immediate	Local Save & Batch	Central Save & Batch
• T Vaginalis (Wet Mount) • KOH Prep • pH • Amine (odor) test • T. Vaginalis Culture (Optional)		• Pap Smear	• Chlamydia (LCR swab)* • CVL Fluid	• B.V. Slide • Syphilis DFA Slide • CVL Fluid • Anal specimens** • Cervical swab #4***

*LCR swabs for Chlamydia are collected only at Visits 1, 2 and 3.
**Anal swabs are collected from HHV-8 positive participants only at Visits 10, 11 and 12 or from anal substudy participants.
***Cervical swabs for HIV RNA quantitation will be collected from Visit 12 forward.

VI. COLLECTION AND PROCESSING OF ORAL HHV-8 SPECIMENS

The WIHS HHV-8 Saliva Collection Protocol will be used; this procedure is based on two reports by WIHS investigator Mahvash Navazesh. Form OP03 will be completed to provide tracking and specimen quality information.

1) Subjects are asked to fast (except water) for one hour prior to the test session.

2) Subjects are asked to remove lipstick with a 2x2-gauze square and any removable dental prosthesis. For stimulated whole saliva collection, if it would be more comfortable for the participant to chew with her removable dental prosthesis, she may go ahead and replace them. However, this should be consistent at every visit; if the participant removes her dental prosthesis at her first Oral HHV-8 visit, she should remove them again at follow-up visits.

3) Subjects rest for five minutes (no talking or reading) before saliva collection begins.

4) Whole-stimulated saliva is collected over **five minutes** by the spitting method using a standard-size gum base as a stimulant. The frequency of stimulation is controlled by a metronome at about 70 chews per minute. The subject is asked to expectorate saliva into the graduated test tube once per minute. (Remind subjects <u>not</u> to spit out the gum base at end of each minute.)

5) Send the specimen to the local lab for processing. As long as the specimen is received by the local lab the same day, it is not necessary for it to be stored or transported on ice. It should get to the local lab the same day as collection and be aliquoted and frozen that day. The maximum waiting time between collection and aliquoting and freezing should be eight hours. If absolutely necessary, the sample can be put in the refrigerator overnight and aliquoted the next morning, but this should be discouraged.

VII. CENTRAL LABORATORY SHIPMENT SCHEDULE AND DIRECTORY

A. SERUM FOR TOXOPLASMOSIS

Serum for toxoplasmosis is collected at original recruit Baseline only. Ship one tube of 0.5 ml serum monthly on dry ice via overnight delivery on Mondays, Tuesdays or Wednesdays to ensure arrival by Thursday. Keep the second tube in local freezer as back-up. Please be advised that the toxoplasmosis central lab will no longer require that an individual requisition form accompany every specimen. A copy of the shipping log will be all that is required for shipment. Sites should not ship specimens that are not included on the logs.

Ship to: Meg Davis
Serology Laboratory
Research Institute, Palo Alto Medical Foundation
860 Bryant Street
Palo Alto, CA 94302
Telephone: (415) 853-4828
FAX: (415) 329-9114

B. SERUM FOR HTLV 1&2

For original recruits, HTLV specimens will be shipped in three batches; on August 16, September 13 and December 6, 1995. For new recruits, HTLV specimens will be batch shipped to repository for redirection to Dr. Hardy's at a later date (refer to section VIII for the definition and procedure on redirection).

Sera must be accompanied by a hard copy inventory of contents (manifest). For new recruits, samples must be accompanied by two manifests, one for the samples that will be stored in the repository repository and one specifically for the HTLV 1 & 2 samples that are to be redirected.

Sera must be shipped in divided boxes (not bagged as a group). For new recruits, sera must be kept in a separate box from other samples being sent for storage in the repository repository. The serum pairs must be shipped, together, as pairs. These measures assure that the laboratory can assess whether sera have arrived intact. The manifests are to be included in a zip-lock plastic bag inside the appropriate specimen box.

Only specimens collected at the Baseline visit (original and new recruits) are to be tested. Ship specimens no later than Wednesday of the scheduled week, on dry ice, via overnight delivery.

Redirect samples from repository to: Dr. William Hardy
c/o Penny Baron
Memorial Sloan Kettering Cancer Center
Schwartz Building, Room 437
1275 York Avenue
New York, NY 10021
Telephone: (212) 639-8391
FAX: (212) 717-3021

C. HSV SEROLOGY (C66)

For original recruits, sera are to be sent frozen on dry ice by overnight courier for receipt on a weekday. For new recruits, HSV sera will be batch shipped to repository for redirection to Dr. Corey's lab at a later date (refer to section VIII for the definition and procedure on redirection).

Redirect samples from repository to: ATTN: Anne Cent
University of Washington Virology Lab
Room G800A
c/o Children's Hospital and Medical Center
4800 Sandpoint Way, NE
Seattle, WA 98105
Telephone: (206) 987-2088
FAX: (206) 528-2793

Billing must be pre-arranged by each site. Contact Sharon Risley at: phone (206) 526-2117, fax (206) 527-3885, or email srisle@chmc.org.

Additionally, reporting and the time frame for completion must be pre-arranged with Dr. Rhoda Ashley-Morrow. She can be reached at: phone (206) 987-2117or fax (206) 527-3885.

Sera must be accompanied by a hard copy inventory of contents (manifest). For new recruits, sera must be accompanied by two manifests, one for the core samples that will be stored in the repository repository and one specifically for the HSV samples that are to be redirected.

Sera must be clearly labeled with WIHS ID and date of specimen collection. Sera must be stored as 0.5 ml aliquots in 1.8 ml externally threaded plastic vials and must be shipped in divided boxes (not bagged as a group). For new recruits, sera must be kept in a separate box from other samples being sent for storage in the repository repository. The serum pairs must be shipped, together, as pairs. To avoid spilling during shipment, tape the divided boxes closed. These measures assure that the laboratory can assess whether sera have arrived intact. The manifests are to be included in a zip-lock plastic bag inside the appropriate specimen box.

D. SUBGINGIVAL PLAQUE SPECIMENS

Ship overnight mail at room temperature to:

Dr. Jorgen Slots
The Oral Microbiology Testing Laboratory
USC School of Dentistry
925 W. 34th Street, Room 4111
Los Angeles, CA 90089
Telephone: (213) 740-3163
FAX: (213) 740-2194

E. ERYTHEMATOUS CANDIDIASIS SPECIMENS

Place the packet with the slides enclosed in the special self-addressed mailing envelope provided. The address of the Oral Pathology Laboratory is:

Oral Pathology Laboratory
56 – 26 Main St.
Flushing NY 11355

When additional smear kits are needed, please contact Dr. Joan Phelan at:

Telephone: (516) 261-4400 ext. 7415
FAX: (516) 266-6020
E-mail: phelan.joan@northport.va.gov

F. ANAL SUBSTUDY DACRON SWABS

Ship all anal substudy specimens to:

Maria Da Costa
UCSF
521 Parnassus, Room C-233
San Francisco, CA 94143-0512
Phone: 415-476-8885
Fax: 415-476-4204 or 415-502-7338
email: dacosta@cgl.ucsf.edu (prefers email to fax)