ALTERNATIVE SPECIMEN SOURCES: METHODS FOR CONFIRMING POSITIVES

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This discussion involves alternative specimens that can be used for HIV antibody testing. There are several types of samples in addition to serum and plasma that have been found to be suitable for HIV antibody testing. This discussion will concentrate on oral mucosal transudate (OMT) and urine. I would like to acknowledge Dr. Toby Gottfried from Calypte for providing me with information and slides on urine testing. Thank you for your assistance, Dr. Gottfried.

Dried blood spot testing gained some popularity beginning in 1989 when CDC was conducting the Survey of Childbearing Women. In short, testing dried blood spots is basically equivalent to testing serum. Therefore, I will move directly to discussing the testing of oral fluid and urine. Most of the controversy that has surrounded the use of alternative samples involves the quantity of antibody present in those samples. By far, these samples including both urine and oral fluid, contain less antibody than one finds in serum and plasma. Oral fluid contains approximately 800 to 1,000 fold less antibody than is found in serum and plasma. The antibody concentrations found in urine are even less than that found in oral fluid. However, this is not a barrier to accurate test results. There are now very sensitive assays available that can detect, with great reliability, antibodies present at these concentrations.

(Slide 1) This table is from a paper by John Parry and Philip Mortimer. Looking first at secretions from the parotid gland, which represents pure saliva, you see that this fluid contains very little IgG. But it is important to point out that the OraSure sample does not collect just saliva. The OraSure collector is designed to optimize the collection of oral mucosal transudate (OMT). OMT is a serum-derived fluid that contains a high concentration of IgG. OMT is secreted into the mouth by a variety of routes. Again the pure saliva secreted by the parotid gland contains only 0.2 mg/L of IgG. Common oral fluid expectorated into a cup contains an average of 12.1 mg/L of IgG. Examining commercially available collection devices, it is seen that they yield variable amounts of IgG. The IgG concentration by OraSure was higher than that collected by any of the devices. Although, these IgG levels are much less than one finds in serum, Mortimer and Parry point out that only 0.5 mg/L of IgG is required to have a very sensitive test for HIV.

The OraSure device is the only FDA approved collection device for HIV testing. It enhances the collection of oral mucosal transudate and collects a sample with higher concentrations of antibodies than is found in a sample spontaneously expectorated into a test tube. It is a very simple device and collecting the sample is very easy. One simply places the cotton pad between the cheek and the teeth,gently moves it back and forth a couple of times until it is moist, and then holds it in place for two minutes. Following the two minute collection, the top from the transport tube containing the blue fluid is removed, snap off the stick, put the cap back on, and ship it to the laboratory for testing.

One important advantage of OraSure is that the sample is stabilized by the preservative fluid

present in the transport vial. The next slide shows a comparison of the rate of degradation of IgG in the OraSure sample versus a saliva sample expectorated into a tube (Slide 2). The IgG levels in the saliva sample were close to zero after seven days. Even more significant is that in one day you lose more than forty percent of the IgG. This loss is due to the proteolytic activity of the proteases produced by bacteria present in the oral fluid. The OraSure preservative prevents bacterial growth and the subsequent secretion of proteases into the sample. Our current data show that the OraSure sample is stable at temperatures between 4^0C and 37^0C for up to 21 days.

Again, I want to emphasize that we are testing antibodies derived from OMT present in oral fluid. The antibodies that are present in oral fluid come from blood by transudation. The testing algorithm used to test this fluid is the same as that used to test serum and plasma (Slide 3). You screen OraSure samples using an enzyme-linked immunosorbent assay (ELISA). Samples that are repeatedly reactive by ELISA are then tested in the Western blot assay. The reporting is the same as that used to report results obtained by testing serum or plasma. In fact, the Western blot interpretation criteria are the same as those recommended by the ASTPHLD and the CDC- for serum Western blots.

We have done a number of clinical trials at Epitope in our various quests for FDA approval of different oral fluid-based products. We have tested more than ten thousand people in various clinical trials. In those trials, we have looked at all of the usual populations. As a result of those trials, the FDA has approved the HIV 1 ELISA manufactured by

Organon Teknika Corporation, the OraSure HIV 1 Western blot, and the OraSure HIV I Oral Specimen Collection Device. It is now possible to complete the entire recommended testing algorithm using FDA approved reagents.

Let us examine the performance of the OraSure Testing System (Slide 4), looking first at the testing of HIV negative people. In the OraSure HIV 1 clinical trial, 2,897 low risk individuals, mostly blood donors, were tested. The ELISA was negative for 2,880 of these subjects. Seventeen were repeatedly reactive and went on to be tested by Western blot. Of these seventeen, thirteen were negative by Western blot and four were indeterminate. There were no false positives in the study. OraSure correctly identified as negative 2,893 of the 2,897 confirmed negative people. While there were false positives in the study I described, oral fluid, like any other clinical sample, is not impervious to false positive results. Any test presented as 100% sensitive and 100% specific should be viewed with some skepticism. It is unlikely that these numbers will be obtained when the tests are put into the field and used to test thousands of subjects. Clinical trial data provide a good guideline for expected performance, but, field-testing is the most effective final test.

Technical errors are by far the leading source of incorrect results. It is not that the people performing the assays are not skilled and conscientious, it is just that they are performing large volumes of tests so there are many opportunities to make mistakes. Much of the testing is manual and technical errors can occur. Most of you know what types of errors fall into that category so I will not list them.

In the OraSure Western blot clinical trial, we tested 673 people who were seropositive (Slide 5). This group included people with AIDS, people at high risk for HIV, and a number of other sources of samples from positive people. One subject in this group tested negative by ELISA but, was an AIDS patient. The serum Western blot for this person had gp160 and gp24 bands only. The OraSure HIV 1 Western blot showed only a gp160 band. None of the remaining 672 specimens that were tested by the OraSure Western blot were falsely negative, seven were indeterminate and 665 were positive.

Being oral fluid, a common question is what effect on the sample does recent eating, smoking, or drinking have on the test results? Of course, the FDA wanted this same information. We have examined this question extensively. We looked at these questions and also the effect of oral pathology. Sometimes bits of food and tobacco juice contaminate the sample. However, none of these contaminates has any effect on test results.

As with any type of sample, it is possible that a very small number of OraSure samples will be falsely negative. Some of the reasons for false negative ELISA results are well known and are the same as other sample types. Inthe case of OraSure, it is possible the IgG levels in a particular collection are insufficient to give good results. There are other reasons for ELISA false negative results. One is the window period. Following exposure, most people do not become seropositive for 21-28 days. In some cases, the window period may be as long as three months. Genetic diversity can also account for false negativity in certain assays. While genetic diversity does not yet appear to be a significant problem in the U.S., two cases of HIV O infections have been identified.

Like all manufacturers, Epitope and Organon Teknika are constantly trying to improve our products. We have submission for the next generation of ELISAs that has languished at FDA for the past 14 months. This ELISA demonstrated improved specificity and sensitivity in our clinical trial. Like every manufacturer, we are trying to improve our product. Perhaps the most significant improvement was seen in specificity. This means that not as many repeatedly reactive specimens from uninfected people will need to go for expensive Western blot testing. In our improved ELISA clinical trial, we tested 700 people who were seropositive (Slide 6). All 700 were all positive in the investigational oral fluid ELISA. We are hopeful the FDA will conclude their review soon so that we can get this assay into the field. As you know, everybody wants the latest thing, but it is not an easy task for manufacturers to get these things into the field. We also looked at some seroconversion panels and again found that the investigational ELISA was more sensitive than the current product. These data show that the investigational ELISA became positive at the same time or before the serum Western blot became positive. This test shows goodsensitivity for picking up people who are undergoing seroconversion.

We also tested 1,892 low risk individuals, mostly blood donors. All 1,892 were negative by the investigational ELISA for a 100% specificity. (Slide 7)

(Slide 8) This figure shows some examples of the current serum and oral fluid Western blot tests. The pairs of blots represent serum and oral fluid that were collected from the same people at the same time. I want to point out that in some cases the oral fluid Western blot looked stronger than the serum Western blot. In some cases the serum Western blot looked a bit stronger. In other specimens the number and intensity of bands are comparable. The interpretations of both blots were identical. Like the urine Western blot, that I will talk about in a moment, the rate of indeterminate Western blots is less for oral fluid than for serum.

(Slide 9) This shows data from a field trial of oral fluid testing that was done by Tim Granade, Bharat Parekh, and Susan Phillips of the CDC . This was a large study of 4,444 specimens that was performed in Trinidad and the Bahamas (Slide 10). The study evaluated several ELISA and Western blots performed on OraSure samples and compared those results to matched serum specimens (Slide 11) and again the results were quite good! The ELISAs evaluated were GACELISA from Murex and two ELISAs from Organon Technika. The second assay, designated OTC-Mod, was a serum assay that had not yet been modified for use with oral fluid. For that

assay the results were not as good as they might have been had the assay been modified to test oral fluid samples. The sensivity andspecificity of the various assay were as follows: GACELISA 100%, 99.8%; OTC-OF 99.2%, 99.2%; OTC-Mod 99.8%, 100%.

(Slide 12) This slide presents the Western blot results. The prevalence of most individual bands observed on the oral fluid blots were very similar with that observed on the serum Western blots. Although, the frequency of p17, p24, and p55 were less than observed with serum blots, this did not appear to have a detrimental effect on the final blot interpretation.

Now let us examine urine for HIV antibody testing. Like Epitope, Calypte has performed two clinical trials. The first clinical trial was performed to evaluate the performance of their ELISA. The second, for the evaluation of the urine Western blot. The first trial tested approximately 10,000 matched urine and serum specimens, including 1,111 positive specimens. The positive group included AIDS patients, symptomatic HIV postive subjects, asymptomatic HIV positive subjects, and unclassified HIV positive subjects. The sensitivity for the ELISA was determined to be 99% for AIDS subjects and 98.7% overall. There were 14 false negative specimens (Slide 13). Based on a study population of 7,082 paired urine and serum specimens from low risk subjects, the specificity was determined to be 99.14%.

In the second clinical trial, 515 low risk subjects were tested by both the urine ELISA and the urine Western blot. The urine test results were compared to Western blot performed on paired serum samples. A full 514/515 subjects tested negative as expected by the ELISA. It is also interesting that in this population of low risk subjects Western blotswere performed regardless of ELISA results, and there were no indeterminate results. The next slide represents data obtained when testing high risk populations whose HIV status is unknown (Slide 14). In this population, 391 people were tested by serum Western blot with 17 positives, 230 negatives and 123 were indeterminate results. From these results, 17 people appeared to be HIV 1 antibody positive. The matching urine specimens were tested using the investigational urine HIV 1 Western blot: 20 urine specimens were positive, 369 were negative, and 2 were indeterminate. These data suggest that 3 of the urine blots may be false positives. Again, the small number of urine indeterminates as compared to serum demonstrated the reduction of these types of reactions when using urine specimens for testing. Calypte has stated that the urine samples described as false positive may in fact represent HIV infected people whose urine specimens test positive but who are seronegative. The term “compartmentalized immune response” has been coined to describe this phenomenon. You may or may not accept that explanation, but three subjects had positive urine specimens that were discordant with their serum results. Slide 15 represents data obtained by screening 748 known HIV 1 seropositive subjects. This group included AIDS patients, symptomatic HIV seropositives, asymptomatic HIV seropositives, and unclassified HIV seropositives. One AIDS subject was negative by ELISA and would not have been advanced to Western blot. When all 215 were tested by urine Western blot, 2 AIDS patients tested negative and represented false negatives.

Slide 16 lists data obtained when testing 281 subjects with non-HIV disease but with potentially interfering substances. Subjects included people with autoimmune diseases, kidney disease, liver disease, STDs, urinary conditions, neoplasms, and pregnant women. These urine specimens tested by ELISA were problematic. Assuming all of these people were uninfected, several categories were very reactive by urine EIA. It was demonstrated that 32/50 people with kidney/liver conditions, 22/47 with urinary conditions, 25/63 pregnant women, and 17/35 with neoplasms were repeatedly reactive by the urine

ELISA. Overall, in this group 113/281 (40.2%) were repeatedly reactive by ELISA and would advance to Western blot. Two subjects were urine Western blot false positive (specificity 99.3%) and 11/281 (3.9%) were urine Western blot indeterminate. The remaining 268 were urine Western blot negative.

(Slide 17) The next slide compares the urine Western blot to serum Western blot performed on the same samples. The gp160 of the urine low positive control serves as the reading standard. For a line to be scored as present, the intensity must be equal to or greater than the intensity of the gp160 band of the low positive control. If bands were present but of lesser intensity than the gp160 of the low positive control, the bands would be scored as +/-. If the gp160 band is not visible on the low positive control, the test is invalid. The identity of the bands present on the urine high positive control are essentially identical to that present in serum. The positive control for the Cambridge serum Western blot is rather difficult to interpret. The entire strip is dark and it is very difficult to distinguish one band from another. While I have seen strips that look like that I do not believe it is typical. The comparison of matched urine and serum blots collected at the same time from the same subjects show that banding patterns can bequite different. However, the bands present on the urine blot are clear, distinctive, and easy to read. One of the specimens has only a gp160 band and its intensity is greater than that on the low positive control.

The interpretative criteria for the urine Western blot test are shown on Slide 18. Again, the gp160 on the low positive controlis the intensity marker. In the absence of any bands, the urine specimen would be scored as negative. If any bands are present that do not meet the positive criterion, the strip is interpreted as indeterminate. The most notable change from the CDC/ASTPHLD serum interpretive criteria is that the presence of gp160 only can be scored as positive.

Slide 1

IgG Concentrations in Oral Specimens

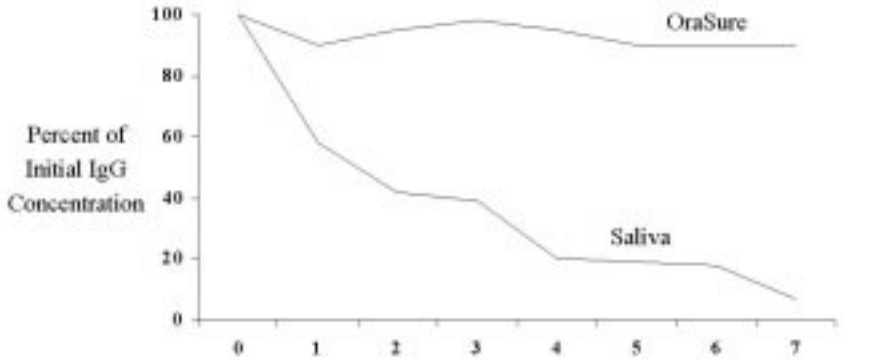
Oral Specimen	Number Assayed	Range (mg/L)	Mean (mg/L)
Parotid gland secretion	-	-	0.2
Saliva (common oral fluid)	19	1.8-48	12.1
Commercial Devices*: Salivette™	228	0.1 - 50	9.6
Omni-SAL™	196	0.3 - >100	23.5
OraSure®	148	7.2 - >100	40.5

*Unpublished data of Brown, DWG, Richards A, and the authors

Source: Modified from Mortimer PP, Parry JV. Clin Diagn Virol. 1994; 2:231-243.

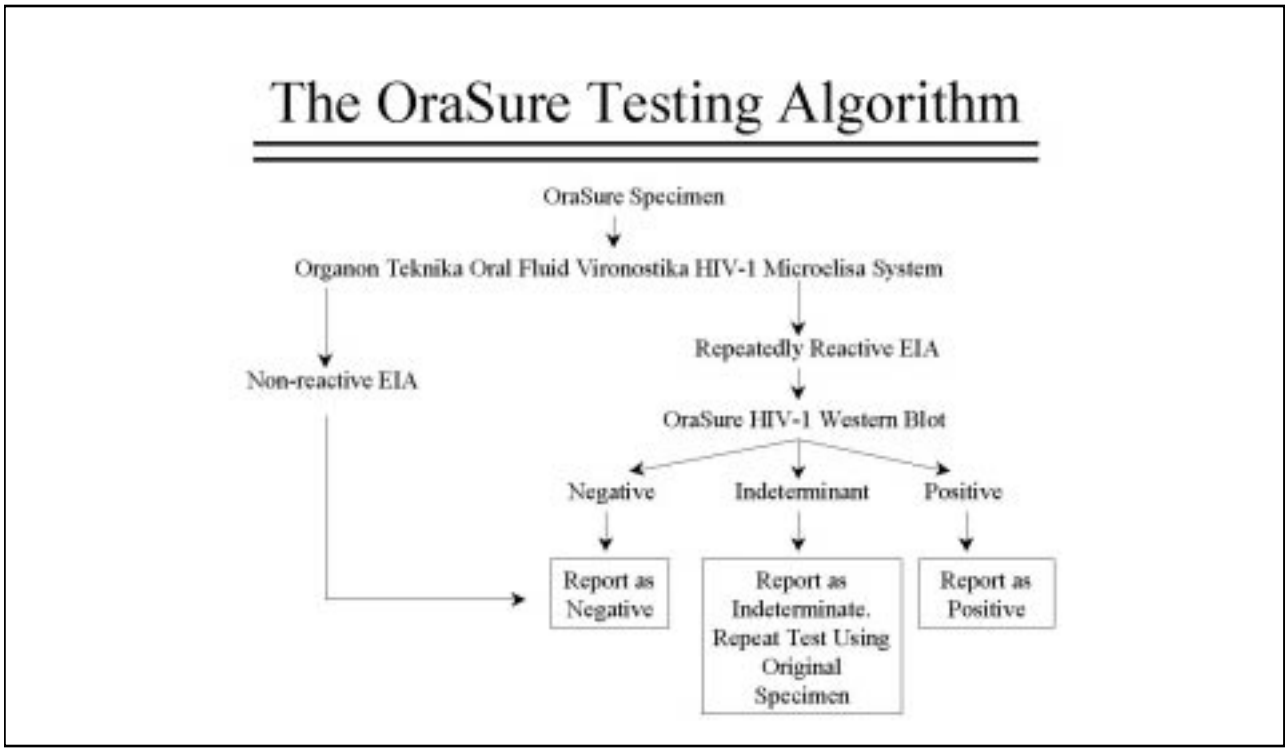
Slide 2

IgG Stability: OraSure Specimens vs Saliva



OraSure specimens are stable for 21 days at 4 to 37°C.

Slide 3



Slide 4

Results of OraSure Testing in Known HIV Negative People

	EIA Repeat Reactive (17)			
Number Tested	**EIA Negative**	**WB Negative**	**WB Indeterminate**	**WB Positive**
2,897	**2,880**	**13**	**4**	**0**

OraSure testing correctly identified 2,893 of 2,897 confirmed HIV negative people as HIV negative.

Slide 5

Results of OraSure Testing in Known HIV Positive People

	EIA Repeat Reactive (672)			
Number Tested	**EIA Negative**	**WB Negative**	**WB Indeterminate**	**WB Positive**
673	**1**	**0**	**7**	**665**

OraSure EIA testing correctly identified 672 of 673 confirmed HIV positive people as EIA repeatedly reactive. On Western blot 665 were positive, 7 were indeterminate.

Slide 6

Summary of Test Results for HIV Positive, AIDS Subjects, and High-Risk Positive Subjects

		Investigational Oral Fluid EIA		Licensed Oral Fluid EIA		Licensed Serum/Plasma EIA		OraSure Western Blot				Serum/Plasma Western Blot			
Risk Group	# of Subjects	NR	RR	NR	RR	NR	RR	Pos	Ind (Viral)	Ind (NVB)	Neg	Pos	Ind (Viral)	Ind (NVB)	Neg
Pos	357	0	357	1	356	0	357	352	5	0	0	356	1	0	0
AIDS	76	0	76	0	76	0	76	76	0	0	0	76	0	0	0
High	17	0	17	0	17	0	17	17	0	0	0	17	0	0	0
Pos	250	0	250	0	250	0	250	246	4	0	0	250	0	0	0
TOTAL	700	0	700	1	699	0	700	691	9	0	0	699	0	0	0

Slide7

Summary of Test Results 1,892 HIV-Negative Subjects

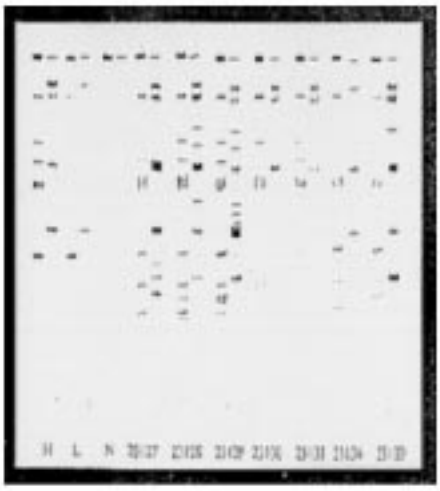
	Investigational Oral Fluid EIA			Licensed Oral Fluid EIA			Licensed Serum/Plasma EIA			OraSure Western Blot					Serum/Plasma Western Blot				
Risk Group	#	NR	RR	#	NR	RR	#	NR	RR	#	Pos	Ind (Viral)	Ind (NVB)	Neg	#	Pos	Ind (Viral)	Ind (NVB)	Neg
Low	504	504	0	504	501	3	4	4	0	4	0	0	0	4	4	0	0	1	3
Low	1,008	1,008	0	1,008	996	12	12	12	0	12	0	1	2	9	12	0	1	4	7
NS	198	198	0	198	196	2	4	4	0	4	0	1	0	3	4	0	0	1	3
High	85	85	0	85	80	5	85	85	0	5	0	0	0	5	5	0	0	0	5
High	97	97	0	97	96	1	97	97	0	1	0	0	0	1	1	0	0	0	1
TOTAL	1,892	1,892	0	1,892	1,869	23	202	202	0	26	0	2	2	22	26	0	1	6	19

* *Total includes three samples in which the laboratory mistakenly performed plasma ELISAs on specimens that were concordantly nonreactive by licensed and investigational oral fluid ELISAs.*

Slide 8

OraSure HIV-1 Western Blot:

Comparison to Serum Blots



Incidence and Intensity of bands on OraSure blots and serum blots are highly correlated.

Slide 9

CDC Oral Fluid Study - Trinidad and The Bahamas

- Granade TC, Phillips SK, Parekh B. Detection of antibodies to human immunodeficiency virus type 1 in oral fluids: A large scale evaluation of immunoassay performance. Clin Diagnostic Lab Immunol. 1998;5:171-175.
- A comparison of serum and oral fluid serologic methods using paired oral fluid (OraSure) and serum specimens collected at the same visit.
- Oral Fluid EIAs = Murex GACELISA, Organon Oral Fluid Vironostika HIV-1 Microelisa, Vironostika HIV-1 Microelisa.
- Serum EIA - Abbott AB HIV-1/HIV-2 (rDNA).
- Western blot - Cambridge Biotech (serum), CDC in-house (oral fluid).

Slide 10

CDC Oral Fluid Study - Trinidad and The Bahamas

Comparison of Oral Fluid and Serum Western blot

- N = 474 matched serum and oral fluid sample
- 474/474 (100%) were serum Western blot positive
- 473/474 (99.8%) were oral fluid Western blot positive
- 1/474 (0.2%) were oral fluid Western blot indeterminate
- Discordant: serum = gp160, p24, oral fluid = gp160

Slide 11

CDC Oral Fluid Study - Trinidad and The Bahamas

Sensitivities and Specificities of serum EIA versus oral fluid EIAs versus serum Western blot

N = 4422

	Number of Specimens					
Serum WB	GACELISA		OTC-OF		OTC-Mod	
	P	N	P	N	P	N
Positive	474	0	470	4	468	6
Negative	8	3,940	33	3,915	0	3,948
Sens.	100%		99.2%		98.8%	
Spec.		99.8%		99.2%		100%

Slide 12

CDC Oral Fluid Study - Trinidad and The Bahamas

Banding Patterns

N = 474 — No. (%) of HIV antibody-positive specimens

	Serum	OF
Gp160	474 (100)	474 (100)
gp120	466 (98.3)	470 (99.2)
p66	469 (98.3)	462 (97.5)
p55	380 (80.1)	365 (77.0)
p51	465 (98.1)	451 (95.1)
gp41	471 (99.4)	459 (96.8)
p31	457 (96.4)	542 (95.4)
p24	469 (98.7)	431 (90.9)
p17	435 (91.8)	267 (56.3)

Slide 13

Sensitivity in AIDS Patients and Seropositive Individuals

Group	Number of Specimens	Urine EIA Results RR	NR
AIDS	300	297 (99.0%)	3 (1.0%)
HIV-1 Positive Symptomatic	275	271 (98.5%)	4 (1.5%)
HIV-1 Positive Asymptomatic	339	332 (97.9%)	7 (2.1%)
HIV-1 Positive Unclassified	197	197 (100%)	0 (0.0%)
Total	1111	1097 (98.7%)	14 (1.3%)

Slide 14

High Risk Populations of Unknown Status

Risk Group	N	Urine EIA RR	Urine EIA NR	Urine Western Blot POS	Urine Western Blot NEG	Urine Western Blot IND	Serum Western Blot[a,b] POS	Serum Western Blot[a,b] NEG	Serum Western Blot[a,b] IND
High	391[a]	90	301	20	369	2	17	230	123

[a]One serum Western blot was unreadable and is excluded from the table

[b]Twenty (20) serum EIA non-reactive specimens were not tested by serum Western blot and are excluded from the table

Slide 15

Confirmed HIV-1 Seropositive Populations

Risk Group	N	EIA RR	EIA NR	Urine Western Blot POS[c]	Urine Western Blot NEG	Urine Western Blot IND	Serum Western Blot POS	Serum Western Blot NEG	Serum Western Blot IND
AIDS	215	214	1	213	2	0	215	0	0
Symptomatic	93	93	0	93	0	0	93	0	0
Asymptomatic	123	123	0	123	0	0	122	0	1[b]
Unclassified	317	317	0	317	0	0	296[a]	0	1[b]
Total	748	747	1	746	2	0	726	0	2

[a]Twenty (20) of the 316 specimens were from Uganda and were not confirmed by serum Western blot, but rather by a second manufacturer's EIA and by agglutination

[b]The specimens did not meet the required band intensity criterion for a Positive serum Western blot result

[c]No intensity criterion associated with the low positive control was used in this analysis

Slide 16

Urine from Populations with Non-HIV Disease and Potentially Interfering Substances

	N	Urine EIA RR	Urine EIA NR	Urine Western Blot POS	Urine Western Blot NEG	Urine Western Blot IND
Autoimmune	25	7	18	0	25	0
Kidney/Liver	59	32	27	0	55	4
STD	37	5	32	0	37	0
Urine Cond.	47	22	25	1	45	1
Pregnant	63	25	38	1	59	3
Neoplasms	35	17	18	0	35	0
Multiple Trans.	13	5	8	0	10	0
Multiparous	2	0	2	0	2	0
Total	281	113	168	2	268	11

Slide 17

Interpretation Of Urine Western Blot

Intensity of Band	Reactivity Score
Absent	Neg
Less than the intensity of the gp160 band on the Low Pos Urine control	+/-
Greater than or equal to the intensity of the gp160 band on the Low Positive Urine control	Pos

Slide 18

Interpretation of Urine Western Blot

Pattern	Interpretation
No bands present	Negative
Any bands present but pattern does not meet criterion for POSITIVE	Indeterminate
The gp160 band POSITIVE. Other bands may or may not be present	Positive