

CONSENSUS PROTOCOL FOR THE COLLECTION, PROCESSING, AND TESTING OF SEMEN

1. PRINCIPLE AND CLINICAL SIGNIFICANCE

- 1.1 Semen is the major vehicle for the sexual transmission of HIV-1. The ability to isolate infectious HIV from the semen and to quantitate viral burden in the form of cell-free or cell-associated HIV-1 RNA in semen are important for epidemiologic and public health aspects of the epidemic. Earlier studies used viral culture to detect HIV in semen. Cell associated culturable virus recovery rates ranged from 8 to 55% (1, 10, 15-19). Much lower recovery rates (3-15%) were reported by these investigators for cell-free seminal-plasma viral load (>3.5 to 4 log), and an AIDS diagnosis was more apt to have positive seminal cell HIV cultures.
- 1.2 More recently, quantitative HIV RNA and DNA assays have been employed (4, 5, 8, 9, 12, 14, 17, 19, 21). Overall these studies have demonstrated that 60-75% of men shed HIV RNA in the seminal plasma and that 65-80% have detectable HIV DNA in seminal cell pellets. Recent cross-sectional studies using commercially available RNA kits have concluded that seminal plasma RNA levels are significantly correlated both with blood plasma RNA levels (8,19), and the recovery of infectious virus from seminal cells (4, 19) but not with CD4 cell count (4, 8, 19), stage of disease (4, 19) or antiviral therapy (4, 8, 19).
- 1.3 Knowledge of the effect of topical microbicides and/or antiretrovirals on reduction of viral load in semen is necessary for the evaluation of compounds that may be useful in prevention of transmission. It has been demonstrated in longitudinal studies that the amount of HIV RNA in seminal plasma increases with time in individuals who progress to AIDS (7) and decreases with effective antiviral therapy (7, 8). Antibiotic treatment of pathogens causing urethritis, especially gonorrhea, can also reduce seminal plasma viral load (3). In addition, since the viral burden in genital secretions may serve as a reservoir in patients who have had their virus seemingly eliminated from the peripheral blood, the quantitation of virus in semen has become a major focus of clinical trials. The following procedures can be used for conducting studies involving semen.

2. SPECIMEN REQUIREMENTS

- 2.1 Patient Preparation and Specimen Collection
 - 2.1.1 The subject should refrain from sexual activity for at least 48 hours prior to donation.
 - 2.1.2 The subject should wash his hands and penis and then use an antiseptic towelette to wipe the head of the penis including the opening. If the subject is uncircumcised, the foreskin should be pulled back before cleaning the head and opening.
 - 2.1.3 The subject should masturbate and collect the specimen in a sterile container, for example a sterile urine collection container. The time that the specimen was produced should be recorded on paperwork accompanying the specimen.

2.2 Specimen Transport

- 2.2.1 The container should be placed in a zip-lock bag and then in an appropriate transport carrier. The mode of transport will determine the type of carrier required. For example, specimens transported by cab must comply with DOT regulations for infectious substances. Effort should be made to keep the specimen container upright.
- 2.2.2 The specimen should be rapidly transported at room temperature to the clinic or directly to the lab. The laboratory should receive the specimen within 2 hours if possible.

3. CULTURE OF SEMINAL CELLS

3.1 Reagents

- 3.1.1 Note: all reagents should be prepared using TYPE 1 reagent grade dH₂O.
- 3.1.2 Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS): Store at room temperature. Note manufacturer's outdate or discard one month after opening.
- 3.1.3 Penicillin-Streptomycin: Available as 100,000 units/mL penicillin and 100,000µg/mL streptomycin. Open bottle under laminar flood hood only. Divide into 0.6mL aliquots in sterile 1.5mL microfuge tubes. Store unopened bottles and aliquots at -20°C, labeled with a 1 year outdate.
- 3.1.4 Nystatin: Available as 10,000 units/mL suspension. Open bottle under laminar flow hood only. Divide into 2.1mL aliquots in sterile snap-cap tubes. Store unopened bottles and aliquots at -20°C, labeled with a 1 year outdate.
- 3.1.5 Fetal Bovine Serum (FBS): available in 500mL sterile bottles from various manufacturers. Store frozen at -20°C. Note manufacturer's outdate. If it is not heat inactivated by the manufacturer, then rapidly thaw a bottle in a 37°C water bath, then heat-inactivate in a 56°C water bath for 30 minutes with occasional shaking. The level of the water in the water bath should be as high as the level of the serum in the bottle. Store at 4°C after thawing. Thawed heat-inactivated FBS has a one month outdate. (Note: FBS lots other than those that are part of the ACTG reserve should be pretested at the site for toxicity to normal human lymphocytes.)
- 3.1.6 RPMI 1640 medium with L-glutamine (2mM): Store at 4°C and observe manufacturer's outdate.
- 3.1.7 IL-2 (interleukin-2): Note the manufacturer's outdate and storage requirements. As needed, thaw a 50mL bottle and freeze the remaining unused portion.

- 3.1.8 Viral transport medium (VTM): To make 100mL with the final concentrations of 1,000 units/mL penicillin, 1000µg/mL streptomycin, 200 units/mL nystatin:
 - 3.1.8.1 To 97mL of RPMI 1640 medium with L-glutamine, add 1mL penicillin/streptomycin.
 - 3.1.8.2 Add 2mL nystatin solution.
 - 3.1.8.3 Dispense in 10mL aliquots. Store at 4°C for up to a month.
- 3.1.9 Seminal cell culture medium: To make 100mL with the final concentrations of 500 units/mL penicillin, 500µg/mL streptomycin, 100 units/mL nystatin, 20% FBS, 5% IL-2:
 - 3.1.9.1 To 73.5mL RPMI 1640 medium with L-glutamine, add 0.5mL penicillin/streptomycin.
 - 3.1.9.2 Add 1mL nystatin solution.
 - 3.1.9.3 Add 20mL heat inactivated FBS.
 - 3.1.9.4 Add 5mL IL-2
 - 3.1.9.5 Store at 4°C for up to 30 days.
- 3.1.10 Trypan Blue Stain: This stains non-viable cells dark blue and is used to determine viable cell-count (viable cells will be clear).
 - 3.1.10.1 Prepare a 0.4% solution by adding 0.4g Trypan Blue and 1mL Glacial Acetic Acid to 9mL distilled water or saline.
 - 3.1.10.2 After dissolving, filter solution through a Whatman filter paper or 0.45µ filter.
 - 3.1.10.3 Store at room temperature for 6 months.
 - 3.1.10.4 Alternatively, trypan blue can be purchased from Sigma.
- 3.1.11 PHA-stimulated uninfected donor PBMCs-see the Specimen Processing section in this manual for instructions on preparing these cells.

3.2 Equipment and Supplies

- Gloves
- Lab coat or gown
- Sterile cup (such as sterile urine container)
- Sterile 15mL conical centrifuge tubes
- Sterile 1, 5, 10 and 25mL pipettes
- Sterile 5mL tubes
- Hemocytometer

Sterile 24 well tissue culture plate
Sterile 100mL bottle
200 μ L and 1000 μ L pipette tips
Bleach (household bleach diluted 1/100 with tap water)
Laminar flow hood (Class 2 biosafety hood)
Centrifuge capable of speeds up to 800 x g and equipped with a horizontal rotor and O-ring sealed safety cups
Vortex
Microscope
CO₂ incubator (37 $^{\circ}$ \pm 1 $^{\circ}$ C with humidity)
37 $^{\circ}$ C and 56 $^{\circ}$ C water baths
Pipette aid
Microcentrifuge tubes
Microcentrifuge
Fume hood (for preparation of Benzidine solution)

3.3 Procedure

NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING THE USE OF GLOVES AND LAB COATS)

- 3.3.1 Allow liquefaction of semen to occur. This typically occurs within 20-45 minutes of specimen collection
- 3.3.2 Transfer the sample to a conical centrifuge tube using a pipette. Measure and record the volume of semen, noting anything unusual about the sample (i.e. contaminated with RBCs, very viscous, etc.)
- 3.3.3 Centrifuge semen at 600-800 x g for 10 minutes.
- 3.3.4 Remove supernatant, divide in 0.5mL aliquots and freeze at -70 $^{\circ}$ C.
- 3.3.5 Resuspend cells in the pellet from step 3.3.5 in 5.0mL VTM if a culture is anticipated or in HBSS or PBS. (Do not use FBS or amphotericin as these reagents will inhibit HIV culture.) Centrifuge at 400-600 x g for 10 minutes.
- 3.3.6 Note: At this point some investigators remove spermatozoa from the cellular fraction using a standard ficoll-hypaque gradient. This may be important for studies in which HIV proviral DNA is investigated. DNA found in sperm may interfere with the analysis of HIV DNA. If this proves to be a problem, layer the seminal cells onto 4mL of ficoll-hypaque, then centrifuge at 600 x g for 20 minutes. Carefully remove the non-spermatozoal cells found at the interface and resuspend in 10mL HBSS or PBS. Proceed with step 3.3.8 below. If sperm cells do not interfere with your assay, this ficoll separation does not need to be performed.
- 3.3.7 Centrifuge at 400-600 x g for 10 minutes.

- 3.3.8 Repeat the wash step (3.3.6) once and resuspend in 3mL seminal cell culture medium that contains high doses of antibiotics (penicillin at 500u/mL, streptomycin at 500µg/mL, and nystatin at 100u/mL). Note: Gentamycin may be substituted for streptomycin.
- 3.3.9 Determine the viability of the cells using trypan blue dye exclusion method (see the Specimen Processing section in this manual for procedure). An accurate cell count of the white blood cells will be difficult as they cannot be readily distinguished from immature germ cells. The number of cells will vary with each specimen. Record this number such that it may be retrieved if needed.
- 3.3.10 Use half of the suspension (i.e. 1.5mL from step 3.3.9 for the quantitative microculture by adding it to an additional 1.5mL seminal cell culture medium and then making 5 fold dilutions in seminal cell culture medium.
- 3.3.11 Proceed with the quantitative culture using the ACTG consensus protocol (except calculate the Infectious Units per Ejaculate by multiplying the IUPML by 6, since 1/6 of the total ejaculate cells were used in each duplicate A well of the quantitative culture.
- 3.3.12 Alternatively, use the 1.5mL of diluted semen from step 3.3.9 to set up a qualitative HIV microculture using the ACTG consensus protocol found in the Virologic Methods section of this manual. Adjust the volume of donor cell solution to 1.25mL per well.
- 3.3.13 If a culture is positive, save viral isolates as described in this Virologic Methods/Quantitative PBMC microculture section of this manual.
- 3.3.14 Freeze 2 aliquots of primary seminal cells/patient ejaculate:
 - 3.3.14.1 Centrifuge the remaining primary seminal cells for 10 minutes at 600-800 x g.
 - 3.3.14.2 Remove the supernatant and then resuspend the remaining seminal cells in 2mL cryoprotective medium (DMSO containing freezing medium).
 - 3.3.14.3 Divide into two 1.0mL aliquots and freeze following the procedure found in the Specimen Processing/PBMC Cryopreservation section in this manual.
 - 3.3.14.4 Store in gas phase of LN₂.

4. QUALITY CONTROL

PHA stimulated donor cells used for culture should always be checked to confirm that the donor is HIV-1 negative or that contamination has not occurred.

5. SEMINAL PLASMA RNA ASSAY

5.1 There are probably as many versions of this as there are investigators working in the field. One thing is clear: there are factors in seminal plasma that inhibit the PCR reaction unless they are removed. The standard Roche RNA and Roche ultrasensitive extraction methods do not remove these inhibitors (4, 5, 8). Options are to use the boom silica bead extraction assay (2), bioMerieux's Nuclisens RNA assay which includes the silica bead extraction procedure, or pellet the virus using ultracentrifugation.

5.1.1 Specimen Preparation

5.1.1.1 Allow liquefaction of the semen to occur. This typically occurs within 20-45 minutes of specimen collection.

5.1.1.2 Transfer the sample to a conical centrifuge tube using a pipette and measure and record the volume of the semen noting anything unusual about the sample (i.e. contaminated with RBCs, very viscous, etc.).

5.1.1.3 Centrifuge 600-800 x g for 10 minutes.

5.1.1.4 Remove supernatant then divide into 0.25mL aliquots and freeze at -70°C.

5.2 Measurement of HIV-1 RNA in Seminal Plasma

5.2.1 BioMerieux

Use bioMerieux's Nuclisens assay following instructions found in the package insert. Also see HIV-1 Molecular Methods found in the Virologic Methods section of this manual.

5.2.2 Roche (boom silica bead extraction required)

See HIV-1 Molecular Methods found in the Virologic Methods section of this manual. Note: the boom extraction standard method is recommended for use with semen as the pelleting step in the boom extraction ultrasensitive method can cause concentration of the inhibitors present in semen that the assay cannot overcome.

6. REFERENCES

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Procedure: ACTG Consensus Protocol for the Collection Processing and Testing of Semen

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