

# Fairfax Cryobank

A Genetics & IVF Institute Cryobank

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August 20, 2004

Division of Dockets Management  
(HFA-305)  
Food and Drug Administration  
5630 Fishers Lane, Rm. 1061  
Rockville, MD 20852

Re: Docket No. 2004D-0193

Draft "Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products"

Please find below our comments regarding the guidance document.

*Item #1*

II.F. What records must accompany the HCT/P after the donor-eligibility determination has been completed?

Under § 1271.55 you must provide the following records with each HCT/P:

- A distinct identification code (such as an alphanumeric code) affixed to the HCT/P container, that relates the HCT/P to the donor and to all records pertaining to the HCT/P and, except in the case of autologous donations or directed reproductive donations, does not include an individual's name, social security number, or medical record number;
- a statement whether, based on the results of screening and testing, the donor is determined to be eligible or ineligible; and
- a summary of the records used to make the donor-eligibility determination.

The summary of records must include:

- a statement that the communicable disease testing was performed by a laboratory or laboratories: (1) certified to perform such testing on human specimens under the Clinical Laboratory Improvement Amendments of 1988 (42 U.S.C. 263a) and 42 CFR part 493; or (2) meeting equivalent requirements, as determined by the Centers for Medicare and Medicaid Services (CMS);
- a listing and interpretation of the results of all communicable disease tests performed on the donor;
- the name and address of the establishment that made the donor-eligibility determination; and
- a statement noting the reason for the determination of ineligibility in the case of an HCT/P from a donor who is ineligible based on screening and released under § 1271.65(b).

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**Response:** This specifies "the results of all communicable disease tests performed on the donor." In the case of reproductive donors this list may be exhaustive and portions irrelevant to the specific specimen being transported. Semen donors donate for a minimum of six months with at least one specimen produced per week, thereby being significantly different than cadaveric donors. Please provide clarification as to required tests. Our recommendation is to limit to applicable tests for the particular specimen as provided in II.C.1

*Item #2*

II.J How do I store HCT/Ps from a donor who has been determined to be ineligible? Under § 1271.65(a), if a donor is determined to be ineligible, you must store or identify the HCT/Ps from the ineligible donor in a physically separate area clearly identified for such use, or follow other procedures that are adequate to prevent improper release, until the HCT/Ps are destroyed or distributed for use in certain limited circumstances identified in § 1271.65 (b) and (c), described in Section VII.B. of this document.

FDA believes there are a number of ways in which you may comply with this requirement. Examples include employing separate refrigerators or freezers, using separate shelves in a single refrigerator or freezer, and using an automated designation system. Since § 1271.47(a) requires you to establish and maintain procedures for complying with these donor eligibility regulations, you should describe the method you choose to comply with this regulation in your standard operating procedures (SOPs).

**Response:** We request clarification as to the definition of "ineligible" in paragraph 1. This appears to refer to a donor as opposed to a particular specimen or group of specimens from that donor. An example is a semen donor who has contracted *Chlamydia*, thereby making suspect specimens ineligible, but the donor himself would be able to continue donating upon appropriate medical treatment and reentry protocols.

*Item #3*

III.E.16. persons who have had both a fever and a headache (simultaneously) during the 7 days before donation (Ref. 8), we recommend that:

- The donor be deferred from donation; or
- The donor be deferred for 28 days after the interview for living donors who may donate at a later date.

**Response:** We request guidance on how to handle with semen donors who donate repeatedly over a period of 6 months or more. If the donor has no other apparent risk factors for West Nile Virus, asking this at every donation for gamete donors who are quarantined for a six month period is excessive and burdensome.

*Item #4*

IV.B. What type of test must I use?

You must use an appropriate FDA-licensed, approved, or cleared donor screening test (if applicable to your HCT/P and available) in accordance with the manufacturer's instructions to "adequately and appropriately" reduce the risk of transmission of the relevant communicable disease agent or disease. (§ 1271.80(c)).

- FDA recommends that you choose a test that is the best available for the purpose of reducing disease transmission. Over time, as new and improved tests are made available, older tests may no longer adequately and appropriately reduce the risk of disease transmission. We list in section V tests that we consider to meet this requirement as of the date of this guidance.
- We believe that, in some instances, you may need to conduct more than one test to adequately and appropriately test for a single disease agent.

**Response:** Please see attached supporting documentation for *Semen Analysis Protocol Using Nucleic Acid Amplification (NAT)* compiled by Brian D. Mariani, PhD, Laboratory Director of the Genetics & IVF Institute's Molecular Infectious Disease Laboratory (CLIA # 49D0952503). Given the stringent protocols and validation of this laboratory, we recommend the guidelines be revised to require testing be completed using tests kits that are FDA-licensed, approved, or cleared or be performed at a sufficiently validated independent CLIA inspected laboratory utilizing NAT technology.

*Item #5*

IV.C How do I interpret test results?

You must interpret test results according to the manufacturer's instructions in the test kit's package insert (§ 1271.80(c)).

We are aware that some HCT/P establishments rely solely on the test results obtained by an organ procurement organization (OPO). As described by the Centers for Disease Control and Prevention (CDC), an OPO may run an enzyme immunoassay initially in triplicate (Ref. 46). If that is the case, and you are relying solely on such triplicate testing, we recommend that you obtain the results of the three individual tests performed in a single run. FDA believes that, in such a case, three nonreactive results in a single run would constitute a negative test result. If any other results are obtained, the donor would not be eligible to donate.

**Response:** This statement is reasonable and prudent for cadaveric cases in which there is not the opportunity to adequately retest the donor when given an indeterminate or initially reactive test and where a 6-month quarantine period is lacking. However, in the case of semen donors who donate on a weekly basis and can conveniently be retested prior to release of the specimens, this is not a logical ruling. We propose the guidance state that any reactive or indeterminate test must be

repeated with confirmatory testing. We recommend mandating such confirmatory testing in all cases of reactive tests. If a repeatedly reactive specimen is not confirmed positive by confirmatory testing (such as Western Blot analysis following a reactive ELISA screen in the case of HIV 1 & 2) and is subsequently found to be non-reactive by additional testing of at least two subsequent specimens, the individual specimen or specimens affected may be deemed acceptable upon review of the results by the medical director. It is important to remember that most tests have a certain number of expected false positives but this is no reason to exclude living donors who can be retested to confirm non-reactive status beyond a reasonable doubt.

*Item #6*

IV.E When do I collect a specimen for testing?

You must collect the donor specimen for testing at the same time as cells or tissue are recovered from the donor, or, if this is not feasible, within seven days before or after the recovery of cells and tissue (§ 1271.80(b)).

In the case of anonymous semen donors who donate repeatedly, you do not have to test the donor at each donation, but you must test him the first time and at least every six months. You must not release the semen unless you have quarantined it for at least six months, collected and tested a new specimen from the donor, and found him to be negative for all required infectious disease testing (§§ 1271.80(b) and 1271.85(d)).

**Response:** Provided proper screening is completed during the active donation periods, it is essentially irrelevant if the donor has Chlamydia or Gonorrhea 6 months or more after his last donation. The retesting of semen donors for these infectious diseases provides information of limited clinical significance whereas the retesting of semen donors for HIV1/2, HBV, HCV, RPR, and HTLV I/II provides information of valuable clinical significance. The safety of the product is not enhanced by the additional requirement to retest semen samples. We recommend revision of this guideline to require only retesting of “infectious disease agents that cannot be immediately detected after exposure through most currently available blood tests” (Docket 1997N-0484S, HHS Final Rule\*) such as HIV1/2, HBV, HCV, RPR, and HTLV I/II.

\*Per page 146 of the final rule, “We also considered waiving the requirement for semen quarantine and anonymous donor retesting to detect infections during the window period, when a donor’s infection may not yet be detectable by blood tests. However, this alternative would expose recipients and the public to risks from infectious disease agents that cannot be immediately detected after exposure through most currently available blood tests (e.g. tests for HIV and HCV).”

*Item #7*

V.A. *Confirmatory tests:* If you perform a confirmatory test, negative results on a confirmatory test would not override a reactive screening test (except for syphilis).

*Example:* You perform a confirmatory test on a potential donor who has tested reactive for Hepatitis B surface antigen on an enzyme immunoassay. The confirmatory test is negative. Despite the negative confirmatory test, you determine the donor to be ineligible because the screening test was reactive.

**Response:** Please clarify why syphilis is exempt. As addressed in Item #5, we recommend revision to exempt semen donors that are tested repeatedly, quarantined, and retested prior to release.

*Item #8*

V.B. For what additional diseases must I test donors of viable, leukocyte-rich cells or tissue and what tests should I use?

1. You must test donors of *viable, leukocyte-rich* cells or tissue for the following diseases, in addition to those listed in section V.A. (§ 1271.85(b)). You must use an FDA licensed, cleared, or approved test where such a test is available (§ 1271.80(c)).

We recommend that you use the tests listed in parentheses:

- a. Human T-lymphotropic virus, types I and II (FDA-licensed screening test for anti-HTLV I/II) (Refs. 60, 61)
- b. Cytomegalovirus (FDA-cleared screening test for anti-CMV).

*Special note on CMV:* CMV is not a relevant communicable disease or disease agent. However, establishments are required to test donors of viable, leukocyte-rich cells or tissues for CMV. A donor who tests reactive for CMV is not necessarily ineligible to donate HCT/Ps. You must establish and maintain an SOP governing the release of HCT/Ps from donors whose specimens test reactive for CMV (§ 1271.85(b)(2)). We recommend that the SOPs be based on current information on the potential for disease transmission from the type of HCT/P to be made available for use and that the SOP limit use of an HCT/P based on the CMV-reactive status of the recipient.

**Response:** The recipient's physician should counsel said recipient on CMV status when selecting a donor and offer testing of the recipient should they select a CMV reactive donor. It is impractical to expect the gamete or tissue bank to assume the burden of patient care and the potential liabilities of providing medical advice. Given the prevalence of CMV in the general population the physician must be the ultimate authority for the use of all HCT/Ps. Per page 100\* Docket 1997N-0484S, HHS Final Rule, we agree with the last statement regarding a physician making an informed decision regarding a particular patient.

\*“One comment asserted that, for reproductive cells, it is unnecessary to require the CMV status to accompany the product, because approximately 40 percent of semen donors are (IgG) positive. The comment noted that it is rare for the physician conducting the insemination to review this information, and that, for

this reason, the information is provided only upon request.” Their response is “We continue to believe that information about the semen donor’s CMV status should appear in materials accompanying the HCT/P, so that physicians may rely on this information to make informed decisions about the use of an HCT/P in a particular patient’s situation.”

*Item #9*

VI.D. What follow-up testing is required for anonymous semen donors?

At least 6 months after donation, you must collect a new specimen from the donor and repeat all testing required under § 1271.85(a) through (c) (§ 1271.85(d)). You must *quarantine* the donated semen until the retesting is complete and the donor is determined to be eligible.

*Example:* A donor tests negative for HBsAg and Hepatitis B core antibody. He is retested 6 months later, and is still negative for HBsAg, but is reactive for Hepatitis B core antibody. The donor is ineligible.

**Response:** We oppose the testing requirement for repeated *Chlamydia trachomatis* and *Neisseria gonorrhoea* testing. These tests are not antibody tests and we recommend that if testing is completed on or after the last specimen that no repeat testing is required. Completing these tests six months after the last donation provides little to no relevant information regarding the safety of the quarantined specimen. We also recommend policy development for scenarios such as the one posed in the above example for protocols to follow when a donor is determined ineligible. It would be most beneficial to establish a clear differentiation between donor ineligibility and specimen ineligibility in the case of gamete donors. An example is a donor who has been active for a one-year period, thereby having 6 months of specimen availability (i.e. specimens having completed a 6 month quarantine and retesting). Suppose this donor is found to be reactive for *Chlamydia trachomatis* at 6 month testing after his last donation (18 month from his first donation). He already has 6 months of specimens from months zero through five that are available for public distribution, months six through twelve are in quarantine and per the guidelines as written would be ineligible, despite the fact that they had been tested for *Chlamydia trachomatis* at the time of donation. A six-month quarantine for *Chlamydia trachomatis* and *Neisseria gonorrhoea* is not necessary as there is no period for seroconversion in these tests. In the above scenario with the current guidelines the donor is ineligible. Thereby, specimens that have already been available would be ineligible and require a recall.

*Item #10*

VII. EXCEPTIONS

This section describes: (1) situations when you are not required to perform a donor-eligibility determination; (2) situations in which the donor-eligibility determination is incomplete; and (3) situations in which the use of cells or tissue from a donor who has been determined to be ineligible is not prohibited. These situations require special labels. FDA understands the term “label” when used in this guidance and in §§ 1271.60(d), 1271.65(b), and 1271.90(b), to mean either (1) a printed label affixed to the container, or (2) a printed label affixed as a tie-tag to the immediate container of the HCT/P.

**Response:** We recommend “immediate container” be replaced with “shipping container”. In the case of cryopreserved specimens in ampules, vials, or straws it would severely compromise the integrity of the HCT/P to relabel or attach a label directly to the immediate container of semen. Also, the size of such containers poses severe limitations on labeling prior to cryopreservation. For cryopreserved reproductive specimens, the label shall be affixed to the can or liquid nitrogen tank containing the specimen.

We appreciate the opportunity to comment on the draft guidelines and look forward to your response. Although we are not an AATB accredited facility, we follow many of the same guidelines and we would also like to state that we are in support of the AATB Reproductive Council’s request for revisions. Thank you for your consideration.

Sincerely,



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A Genetics & IVF Institute Cryobank



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Enclosure: Semen Analysis Protocol Using Nucleic Acid Amplification (NAT)

**Semen Analysis Protocol Using Nucleic Acid Amplification (NAT)**

**Molecular Infectious Disease Laboratory (CLIA # 49D0952503)**

**Genetics & IVF Institute**

**Brian D. Mariani, PhD, Laboratory Director**

**August 10, 2004**

**The Molecular Infectious Disease Laboratory (MIDL)** is in compliance with the Clinical Laboratory Improvement Amendments Act of 1988, and the new regulations of 2003, (Centers for Medicare & Medicaid Services), section 42 CFR 493, concerning test validation and quality control. In 4 inspections since 1998, the laboratory has been determined to be without deficiencies in all protocols and operational aspects. The following information addresses CLIA'88 section 493.1213 in describing verification and validation of method performance specifications, covering separately nucleic acid extraction and nucleic acid amplification (NAT).

**Background.** The main mission of MIDL is infectious disease testing of acutely and chronically ill patients, many of which are hospitalized. Nucleic acid testing assays have been developed and validated and CLIA certified for the rapid and sensitive detection of 20 clinical pathogens from a wide variety of specimen types. We serve major hospital groups in the Mid-Atlantic States region of the U.S. Because of the success of the patient testing program, we extended the NAT methodology to the screening of egg and sperm donors, using cervical swab, blood, urine and semen. In the case of semen testing it was deemed important to have an assay that was validated for cryopreserved specimen so that retrospective testing could be performed on stored samples.

The testing of cervical swab specimens and semen for infectious agents using NAT was instituted at The Genetics & IVF Institute (GIVF) in 1998 to overcome the recurrent problem of false-negative results for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using culture or immunoassays. These assays were initially performed on semen from male partners of women seeking assisted reproductive services at GIVF, or from infertile men seeking treatment at the

GIVF Andrology Division. Initial comparative studies were performed on fresh semen specimens from GIVF patients, spouses, and potential donors using culture and/or immunoassay, and NAT for Ct, Gc and Mycoplasma. During this study it was demonstrated that low-level infected specimens that were identified as positive by NAT assays developed at MIDL, were testing negative by culture and immunoassays performed at a commercial laboratory. Our physicians, concerned that infected specimens may be missed by conventional testing, instituted the conversion to NAT after the completion of the study. Because of the success of this methodology, NAT was extended to the screening of cryopreserved donor semen. The following section describes the verification and validation process for the Ct and Gc NAT assays for cryopreserved (and fresh) semen. To date, 3,216 semen specimens have been processed and tested for a variety of pathogens, including *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma hominis*, Herpes Simplex Virus 1&2, Cytomegalovirus, and/or Human Papillomavirus, strains 16 & 18.

**Overview of semen testing assay.** The details of the assay will be described in more detail in the following sections. Briefly, the verified assay for Ct and Ng testing consists of testing 0.2 ml of semen in a "nested" multiplex PCR that targets both organisms and a human internal control sequence, the X-chromosome amelogen gene (AmelX), in the same reaction. Semen nucleic acid is extracted using the Qiagen QIAmp DNA kit designed for human tissue processing. Nucleic acid is captured and purified using the QIAmp silica-resin affinity column system. Recovered nucleic acid is subjected to PCR and analyzed by PAGE and SyberGreen staining. All assays are run in duplicate. The detection threshold is 500 target organisms per ml of semen (500 organisms or greater are detected per ml sample with 100% sensitivity). Multiple negative and positive controls are run in parallel with all clinical assays. The use of an internal positive control eliminates false-negative results due to enzymatic inhibition. Positive controls for each pathogenic target test ensure reagent and reaction fidelity.

**Outline.** The summary is divided into the following categories:

1. "nested" multiplex PCR primer design and optimization
2. PCR primer sensitivity and specificity
3. Internal positive control reaction
4. Semen extraction protocol
5. Sensitivity and specificity of microorganism detection from semen
6. Accuracy (degree of conformity of measurement to a standard)
7. Precision (degree of agreement among individual test results under standard conditions)
8. Validation, reference range, interpretation of results

**Primer design and optimization.** MIDL uses "nested" multiplex PCR for the simultaneous detection of multiple microorganisms and viruses, and a human internal positive control sequence, from the same specimen in the same reaction. The MIDL is comprised of a suite of four physically separated workrooms, each dedicated to a specific step in the test process. Room One is a clean room housing all PCR reagents and primers, and containing a laminar flow "clean bench" in which all amplification reactions are prepared, minus template. This room contains no intact DNA of any type, and dedicated gowns, hairnets, shoe covers and gloves are worn at all times, as is the case for each of the 4 rooms. Room Two contains 2 Biosafety cabinets, class II, for specimen preparation. All patient derived nucleic acids are prepared in this room and added to preassembled PCR reactions (from Room 1) at a dedicated workspace. Room Three contains the thermal cyclers and a clean bench for set up of second-round "nested" PCR. Room Four contains electrophoresis stations for high-resolution polyacrylamide gel electrophoresis, using SyberGreen gel staining and Polaroid photodocumentation. A unidirectional workflow is maintained for the entire lab to prevent endproduct contamination. Additional rooms are used for accession databasing, technician desk space, and office space.

For all PCR primer design strategies, genetic sequences are chosen that satisfy the following criteria: 1) they are highly conserved within all strains of the target species, 2) they are part of vital genetic material such that deletion or alteration of the sequence would effect viability, 3) the target sequence is unique to

the target species, thus eliminating cross reactivity, and 4) the primers do not react with any human sequence. The genetic sequence for the detection of *Chlamydia trachomatis* in our assay is the multi-copy cryptic plasmid, pLGV440. This plasmid is present in up to 10 copies per elemental body. This sequence has been shown to provide accurate and reproducible detection in numerous published studies. For *Neisseria gonorrhoeae* detection, the *cppB* gene residing on the plasmid pJD1 was targeted. This sequence has been used in many research studies and satisfies all the above criteria for accurate and reproducible detection. Primers for a given pathogen are also tested for specificity *in silico* using the BLAST function of the National Center for Biotechnology Information. Since extensive genomic sequence information is available for most clinical pathogens, in part or in toto, primer pairs can be compared easily and accurately for potential cross reaction, or lack thereof, to other microorganisms using these NCBI computer algorithms.

All primer optimization experiments follow the same outline. For "nested" PCR, first round (1°) primers are titrated to provide detection of 10 target genomes per reaction, using 0.1 uM primers for second round (2°) PCR. This is accomplished by testing a range of 1° primer pair concentration from 0.02 to 0.14 uM in reaction with 10, 100, 1,000 and 10,000 copies of target genome.

When a 1° primer pair concentration is found that accurately generates the correct PCR product with no background bands, it is tested against an excess of human DNA (up to 100,000 genome equivalents), and in a mixture of genomic DNA from other organisms targeted in the multiplex PCR, or likely to be present in the specimen type to be tested. When it is determined that a given 1° primer concentration (in conjunction with a 2° primer concentration of 0.1 uM) yields the proper PCR product in a background of excess human DNA, it is tested for technical precision. In this experimental design, 96 identical reactions are run comprised of 10 *Chlamydia trachomatis* or *Neisseria gonorrhoeae* genome equivalents in a background of 50,000 to 100,000 genomes of human DNA. If 100% of the reactions do not yield the expected Ct or Ng product, 1° primer concentration is adjusted upward until 100% precision is achieved, without adding background product.

When the primer conditions for each microorganism are optimized, the 1° Ct and Ng primer pairs are mixed, as are the Ct and Ng 2° primer pairs to comprise each multiplex primer mix. Also included in these mixtures are the 1° and 2° primer pairs for the human sequence that is used as an internal positive control target (described below).

An additional criterion that must be met for our experimental design is that all primers (1° and 2°) for all targets must have an optimal annealing temperature of 57° C. In this manner, all reactions regardless of genetic target can be performed in the same thermocycler at the same time under the same conditions. This relatively high annealing temperature also reduces spurious primer hybridization events.

A summary of the primer sequences and the primer concentrations is listed in a following document.

**PCR primer sensitivity and specificity.** Data gained from the above experiments provides information on assay sensitivity and specificity with respect to the performance of the nucleic acid amplification component of the assay. Sensitivity for each assay is 10 target genomes per reaction, based on the precision experiments (in which 100% of reactions must be positive). Specificity is determined in two ways: by demonstration that multiple reactions using Ct and Ng primers against excess human DNA does not yield ambiguous bands leading to false-positives, and that testing the primers against DNA of other related microorganisms does not results in cross reactivity.

As stated, these determinations of sensitivity and specificity relate to the PCR. Clinical sensitivity and specificity are determined at a later step when verification is performed on particular specimen types.

**Internal positive control reaction.** Each assay is monitored using several positive controls. External positive controls are performed to assess reaction reagents including PCR primers, buffers and enzymes. In addition, all reactions contain primers specific for a single-copy, X-chromosome linked human sequence. This internal positive control has played a significant role in eliminating false-negative results. This control allows assessment of nucleic acid extraction efficiency, nucleic acid recovery and fidelity, and amplification reaction performance, since all aspects of the protocol must work correctly for

this reaction to be scored as positive. All clinical reactions must be positive for this human signal before any judgement is made concerning pathogenic detection. Any decrease or loss of the human signal indicates suboptimal assay performance or failure, and reactions are repeated. For semen specimens, an internal control signal is always observed due to the amount of DNA normally found in semen, except in clinical cases in which reduced DNA yields were found in material from aspermic individuals or other cases where seminal output is severely reduced.

In cases where clinical specimens do not contain adequate human cellular material for the internal positive control to be effective, i.e. cerebral spinal fluid, plasma, serum, urine, vitreous fluid, skin swabs etc., a known quantity of purified human DNA is added to the patient extract and this is run in parallel with the "unspiked" assay. If the supplemental DNA does not generate the expected signal, then it is assumed that the specimen was inhibitory and the extract is regenerated or subjected to a "clean-up" protocol and retested.

**Semen extraction protocol.** Initially, an in-house developed extraction protocol was used for the preparation of semen-derived nucleic acid. Due to the sporadic occurrence of sub-optimal reactions, this extraction protocol was abandoned in September 1998, and replaced by a protocol using the Qiagen Mini DNA kit. Over the years, several different Qiagen nucleic acid extraction kits have been routinely used in the lab for blood, urine, and bodily fluids and tissue preparation targeting DNA and RNA pathogens. Qiagen systems have consistently provided superior, high quality nucleic acid preparations free of inhibitory substances and are rigorously quality controlled. I have established a working relationship with their technical support department and have adapted their protocols for several of our esoteric tests.

For semen testing, the Qiagen protocol designed for human cells and tissues was employed. Two protocols are used, one method for viral detection uses whole semen including seminal fluid, and the second method tests for bacterial and cellular targets and uses cellular material after a centrifugation step (wash) through 1X phosphate buffered saline (PBS). This protocol is used for Chlamydia and Neisseria testing and is described briefly below.

The Qiagen QIAmp DNA kit tissue protocol uses 2 detergent buffer systems (ATL and AL) in conjunction with a Proteinase K digestion step. These treatments result in complete cellular lysis and disruption of all gram-negative bacteria and most gram-positive (except some staphylococci species). Pure strains of Ct and Ng from ATCC, diluted to known concentrations, have been subjected to the QIAmp extraction protocol to verify that these target organisms are completely lysed in this protocol. It was determined that a pre-lysis lysozyme step was not required for Ct and Ng extraction (as is included in our Group B streptococcus protocol).

The cryoprotector used in the semen specimens we are working with consists of the following components, in descending order of concentration: glucose, sodium citrate, fresh egg yolk, glycerol, penicillin, streptomycin, and Tris buffer. None of these components, singly or in combination, are inhibitory to the amplification reaction when using Qiagen reagents for specimen processing.

1. A volume of semen ranging from 100-200 ul is added to 400 ul of sterile 1X PBS buffer in a 1.5 ml microcentrifuge tube and centrifuged for 5 min at 5,000 x g at room temperature. After the supernatant is removed, the cellular pellet containing any cell-free or intracellular bacteria (elemental bodies) is resuspended in a 180-ul volume of Qiagen Buffer ATL and mixed by vortexing. This buffer contains SDS and is designed for tissue disruption. Proteinase K is then added to the lysate and the mixture is incubated for 1 hour at 56°C, after which time 200 ul of Buffer AL is added to complete the lysis process at 70°C for 10 min. Proteinase K is inactivated during this incubation.
2. After lysis, 200 ul of 100% ethanol is added and mixed, and the mixture is applied to a QIAmp spin column and the column is centrifuged at 6,000 x g for 1 min at room temperature. This step allows the cellular debris to be spun through the column while total nucleic acids are bound and retained on the resin matrix. The column is composed of a silica resin that has a high binding capacity for DNA and RNA. The column bound

nucleic acid is washed free of additional debris with several wash buffers prior to elution using a QIAmp PCR compatible elution buffer (AE).

3. The elution is accomplished in 100- $\mu$ l buffer. A 10- $\mu$ l volume of this nucleic acid mixture is now used for each 50- $\mu$ l amplification reaction using "nested" multiplex PCR described above. All assays are performed in duplicate. Semen specimens do not need to be "spiked" with exogenous human DNA due to the adequate yield of donor/patient derived material for the internal positive control reaction.

A 200- $\mu$ l volume of semen is used per extraction assay, typically yielding 10-20  $\mu$ g of DNA, representing a 95-98% recovery.

**Sensitivity and specificity of microorganism detection from semen.** To document the ability to detect the presence of Ct and Ng in semen specimens, experiments were performed in which nucleic acids were prepared from cryopreserved semen inoculated with various concentrations of Ct and/or Ng organisms after thawing. Strains of Ct and Ng of known titer obtained from the American Type Culture Collection (ATCC) were used to seed cryopreserved semen obtained from the GIVF Cryobank and fresh semen from the Andrology Division. Semen inoculations ranged from 10,000 organisms/ml to 10 organisms/ml in a 10-fold dilution series and samples were subjected to nucleic acid extraction and PCR amplification. Importantly, initial experiments were performed using inoculated semen samples that were again cryopreserved before analysis to test the ability of the microorganisms to be detectable after the complete cryopreservation process. It was found that the cryopreservation process did not adversely affect subsequent microorganism detection. Cryopreserved specimens known to be positive by culture assays and rejected by the Cryobank were also obtained and tested during the verification process.

Using an amplification reaction with a 10 copy/assay detection threshold on an extract volume corresponding to 1/10<sup>th</sup> the elution volume, the detection threshold with respect to 200- $\mu$ l semen

volume yields a detection of 500 organisms per 1-ml volume of semen. To verify this detection threshold, a minimum of 96 assays were run containing 500 Ct or Ng per ml of semen (or 100 cells/200 ul semen), and a 100% detection rate was achieved, with no failed reactions. In addition, at least 48 assays were performed containing no microorganisms resulting in 100% negative results, corresponding to no false-positives. With the assay as it is validated, 500 Ct elemental bodies and/or 500 Ng cells are detected per ml of semen with 100% sensitivity and specificity.

In total, during the course of the verification experiments many more than 96 positives and 48 negatives are routinely performed for each target and specimen type. However, these numbers are routinely used as the minimum number of tests required for verification, not including the testing of known culture-positive specimens obtained from our physician network.

As of October 9, 2001, MIDL has performed over 1564 semen specimen assays (3128 in duplicate) for bacterial and viral targets. Assays are typically run in batches of 10-12 specimens. Each assay is performed in duplicate and contains multiple positive and negative controls covering extraction efficiency, analyte quality control, contamination control, amplification enzyme activity and electrophoresis molecular weight standards, as mentioned above.

**Accuracy and Precision.** Assay accuracy, as measured as the degree of conformity of measurement to a standard and, clinically, with respect to the ability to rule in or out specific infection, is calculated based on the results of the verification experiments and the ongoing testing program. Since commencing semen testing, we have had no false-negative or false-positive results based on comparison to corresponding clinical data, and positive and negative control reactions. All our positive results for Chlamydia and Neisseria were reported the ordering physicians, and no patients known to be infected based on clinical criteria have to date tested negative by NAT.

Assay precision, as measured by the degree of agreement among individual test results under standard conditions was also determined from the verification data. Numerous experiments performed testing semen with known pathogen titers in multiple assays prepared from the same stock reagents demonstrated the 100% reproducibility of the assay. The precision of the ongoing testing program is calculated by the consistency and signal strength of the internal positive control, and by evaluation of the positive control reactions prepared from quality controlled standards run in parallel with each batch of assays.

**Validation, reference range and interpretation of results.** Assay validation is an ongoing process dedicated to providing information concerning test performance, accuracy and precision. The components of validation are quality control, proficiency testing, employee competency, instrument calibration and clinical correlation of test results. The laboratory and the Institute as a whole abide by strict quality control and quality assurance measures that generally have exceeded CLIA standards. All aspects of laboratory operation are monitored and performance records maintained. All reagents, analytes and standards are maintained and used within assigned shelf lives and according to label instructions. All methodologies involved with nucleic acid extraction, amplification and gel electrophoresis are routinely monitored to document method performance. Any deviations from normal laboratory operations are recorded and discussed in our Institute-wide quality assurance program, and corrective action is taken as appropriate.

The laboratory participates in the College of American Pathologists (CAP) proficiency survey in the areas of sexually transmitted diseases, the Herpes viruses, HIV, Hepatitis virus B & C, HPV, etc. To date we have a perfect reporting record in all categories. The training program for new employees covers an extensive training program and documentation. An internal proficiency testing program is administered to all lab personnel semiannually, consisting of a panel(s) of unknowns prepared by myself or the lab supervisor and performed by the employee during normal testing flow. All instruments and thermometers that require calibration are calibrated according to recommended schedules. Thermocyclers are tested semiannually for precision, reproducibility and block temperature and ramp uniformity.

MIDL works in close association with our own GIVF physicians to correlate laboratory data with clinical findings for patients and donors. Likewise, we communicate closely with the hospital-based physicians and laboratory personnel in correlating NAT data with clinical findings, especially with neonatal testing where correct and timely therapeutic intervention is critical. We have adjusted several of our assay thresholds in consultation with ordering physicians to suit particular clinical needs, such as increased sensitivity for central nervous system infections, and lowered sensitivity to distinguish active CMV infections from latent infections, for example.

The reference range for all the qualitative NAT is NOT DETECTED. As Laboratory Director, I evaluate all NAT results for all assays for both donors and patients. Results are evaluated in light of all negative and positive control data run in parallel before results are reported. Generally, there is concordance between duplicate patient assays. In cases where 1 of 2 tests is positive, the assay repeated, or these results are communicated to the physician with the interpretation that the infection may be low-level, at or below the level of the assays threshold of detection. I am available to all ordering physicians to discuss NAT results, and provide interpretation of the amplification data with respect to clinical findings.