APPENDIX 3D

D.1.GUIDELINES FOR THE DEVELOPMENT OF JOINT WRITTEN AGREEMENTS BETWEEN HISTOCOMPATIBILITY LABORATORIES AND TRANSPLANT PROGRAMS

Histocompatibility testing provides clinicians with data to evaluate the immunological risk of proceeding to transplant. The timing and number of tests may vary depending upon specific needs of the program, waiting times, sensitizing events in individual patients or other considerations. These should be established to best suit the needs and concerns of each transplant program drawing upon the expertise of the histocompatibility laboratory. These guidelines summarize the recommended elements to be included in the joint agreements and provide background and discussion to support the recommendations. Data cited in reviews of histocompatibility testing for renal (1) and thoracic (2) transplantation formed the basis for these recommendations.

The following elements should be included in agreements developed between histocompatibility laboratories and transplant programs:

- A process to obtain accurate and timely history of allosensitization for each patient
- Selection of assay format for antibody screening and for crossmatching
- Selection of timing for periodic sample collection
- Selection of timing for performing antibody screening
- Criteria and a process for establishing a risk category for each patient and crossmatching strategy for each category
- Criteria and a process for use of Unacceptable Antigens or Acceptable Antigens for organ allocation
- Process for monitoring post-transplant or for monitoring desensitization protocols
- Process for ABO verification compliant with Policy 3.1.4 if laboratory is asked to list candidates for its transplant center

History of Allosensitization

It is important to recognize 2 major sources of sensitization:

1. Graft failure – nearly all patients who survive graft failure produce anti-HLA antibodies against mismatched HLA antigens on the failed graft.
2. Previous pregnancies – up to 25% of women who have had children produce antibodies against mismatched paternal HLA antigens. This appears to increase with the number of live births.

Either of these factors raises the strong possibility that a patient has been immunized. Other factors may stimulate antibody production as well (particularly among patients with prior graft failure or pregnancy) including blood transfusions, vaccinations, certain infections and surgeries. Patients with autoimmune diseases (SLE, Age nephropathy) may have autoantibodies that will complicate evaluation as these produce false positive reactions in certain tests. Patients who have any of these risk factors are at high risk of rapidly developing an antibody response on exposure to alloantigens, so it is also important to determine whether any potential sensitizing events have occurred since the patient's antibody status was last tested. Table 1 provides more detail of data to be evaluated in determining sensitization history.

Detection of Alloantibody: Creating an Alloantibody History

Current technologies for antibody measurement offer sophisticated means to detect circulating antibodies, which when evaluated in the context of the patient's sensitization history should provide an estimate of a patient's risk of producing antibody on re-exposure to the specific allogeneic HLA antigens of the donor at the time of transplant.

The major technologies are listed in Table 2. These tests (and others) can be used to assess sensitization in transplant candidates. The strategies should include:

1. Identification of patients who do or do not have circulating alloantibodies to HLA class I and class II antigens.
    a. Initial serial screening should include cytotoxicity and more sensitive tests to identify patients with antibodies.
    b. Several sera should be evaluated to establish a baseline.

2. Characterization of antibody specificity in patients with detectable circulating antibodies using some combination of:
    a. A panel of representative cells for cytotoxicity
    b. ELISA tests for specificity
    c. Antigen-coated microparticles

3. Monitoring patients who do not have antibodies for their development.
    a. Periodic screening of unsensitized patients is important to detect appearance of anti-HLA antibodies.
    b. Characterization of antibody specificity.

The challenge in assessing sensitization status is in evaluating the risk of new patients, previously sensitized patients and patients with low levels of antibodies that are detected only by more sensitive tests (enhanced cytotoxicity tests using anti-human globulin (AHG) or flow cytometry) rather than lymphocytotoxicity. Estimating the risk for patients who have evidence of anti-HLA antibodies that are not detected by cytotoxicity must be accomplished by considering the patient's sensitization history. Antibody titers rise after alloantigen exposure and fall over time when the antigen stimulus is removed, often leaving memory B-cells capable of rapidly expanding and secreting antibodies. The danger is that even the most potent immunosuppressive agents are not effective against a memory response which can increase anti-HLA antibody levels within days after re-exposure to HLA antigens on the graft. Although these antibodies rarely cause hyperacute rejection, they carry a high risk for accelerated acute rejections. Because patients are first encountered and evaluated at different stages of their overall immunological experience, the absence of detectable antibodies does not necessarily mean absence of sensitization. Although obtaining a detailed history of sensitizing events is often difficult, particularly for patients who are geographically distant, clinical transplant programs and histocompatibility laboratories should work together to optimize obtaining this information on a timely basis

Periodic Sample Collection

Monthly serum samples for waiting patients should be collected and maintained by the histocompatibility laboratory to develop an alloantibody history and to facilitate final crossmatches.

Crossmatching Strategies

During the mid-1960's, Terasaki (3) and Kissmeyer-Nielsen (4) independently discovered that preformed anti-donor lymphocytotoxic antibodies caused hyperacute rejection of kidney allografts. Patel and Terasaki reported that 24 (80%) of 30 patients transplanted with a positive crossmatch experienced hyperacute rejection and another 3 lost their grafts within 3 months. Since then a prospective crossmatch has been performed before every kidney transplant with few exceptions and, as a result, hyperacute rejections are rare.

The crossmatch test is a direct test for antibodies against the HLA antigens of a specific donor. Obviously a patient with no history of testing for anti-HLA antibodies cannot be considered to be unsensitized. A patient with broadly reacting circulating lymphocytotoxic antibodies would pose an extremely high risk for a positive crossmatch with a prospective donor. On the other hand, a patient who, after repeated tests against panels of potential donor cells or HLA antigen-coated microparticles or other solid supports, has no detectable circulating anti-HLA antibodies is unlikely to have a positive crossmatch test, assuming that testing was performed against a comprehensive panel of HLA antigens and there have been no intervening allosensitizing events. In the Patel and Terasaki study, only 4 hyperacute rejections occurred

among 168 patients who tested negative against a panel of potential donor cells using a relatively insensitive test. The specific strategies for evaluating the relative risk of an antibody-mediated rejection must be developed through a joint collaboration between the histocompatibility laboratory and transplant program. In thoracic transplantation, prospective crossmatches are not commonly utilized for patients with no detectable HLA antibodies. In renal transplantation, there may be exceptional cases when it would be advantageous to proceed with transplantation before a pre-transplant crossmatch can be completed. However, such cases must be approached with caution to avoid the consequences of unrecognized antibodies (and the underlying immunity they represent) directed against the donor's HLA antigens. In all cases where a pre-transplant crossmatch is waived, a peri-transplant or retrospective crossmatch is recommended to guide post-transplant management. Table 3 lists elements to be included in crossmatching strategies.

References

1. Gebel HM, Bray RA, Nickerson P. Pre-transplant assessment of donor-reactive, HLA-specific antibodies in renal transplantation: contraindication vs. risk. Am J Transplant. 2003 Dec;3(12):1488-500.
2. Reinsmoen N, Zeevi A, Nelson K. Anti-HLA antibody analysis and crossmatching in heart and lung transplantation. Transplant Immunol, 2004 (in press).
3. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. N Engl J Med 1969; 280:735-739.
4. Bergentz SE, Olander R, Kissmeyer-Nielsen F, Olsen TS, Hood B. Hyperacute rejection of a kidney allograft. Scand J Urol Nephrol. 1970;4(2):143-8.

Table 1. Documenting allosensitization

Event	Data	Notes
Previous graft (includes all solid organs and bone or tendon allografts)	Date of transplant, organ(s)	
	Date of graft loss	Dates of graft removal, retransplant, return to dialysis
	Cause of graft loss	
	HLA typing of donor(s)	To aid in interpreting relevance of alloantibody and to identify potential Unacceptable Antigens
	Rejection history, history of delayed function, history of non-compliance or reduced immunosuppression due to infection	
Pregnancy	Number, years of occurrence	Gravida and para
Transfusions	Number, type of product, month and year of occurrence	
Assist device placement	Type of device, date of placement, duration of treatment	Primarily for thoracic transplantation
Disease	Identification of disease(s) causing end-stage organ failure	Autoimmunity may invalidate some laboratory assays
Acute infections	Viral infection or bacterial infection requiring antibiotics	Most important if occurred since last antibody screening test. Induction of cells or antibodies with specificity for HLA, non-specific activation of memory
Chronic infections	Viral infection e.g. HCV	May effect response to tolerance induction protocols
Vaccinations	Type, date of occurrence	Most important for time period since last antibody screening test.

Table 2. Assays to identify alloantibody (antibody screening or crossmatching)

Assay		Description and Use
Standard complement-dependent lymphocytotoxicity (CDC)		to detect IgG antibodies known to cause hyperacute rejection for panel measurements or crossmatch
Anti-human Globulin - enhanced cytotoxicity (AHG-CDC)		to improve detection of weak or low level antibodies for panel measurements or crossmatch
ELISA-based assays		to provide a more sensitive test that does not depend on complement fixation
	Mixed antigens	for monitoring
	Cell equivalents	to measure specificity
	Single antigens	to measure specificity
	Solubilized cells	for crossmatch
Flow cytometry-based assays		the most sensitive test for antibody
	Cell-based	for crossmatch or panel measurements
	Microparticle-based soluble antigens	for panel measurements without background from cell membranes
	Microparticle-based single HLA-antigen beads	for high resolution antibody identification
Determine isotype of antibody		for panel measurements or crossmatches
	IgG or IgM	
	Complement-fixing IgG?	
Rule out contribution by autoantibody		for panel measurements or crossmatches
	Treatment of serum	
	Autologous cells	

Table 3. Recommended elements for crossmatching strategies. Strategies should be tailored to level of risk.

Element	Options
Selection of technique(s)	See Table 2. Level of sensitivity
Selection of serum	Stability of a patient's antibody response incorporated into choice of time interval between serum collection and transplant. Use of historic serum.
Timing	Prior to transplant (number of hours or days) Peri-transplant or retrospective (number of hours or days) Timed to limit cold ischemia

D.2. Histocompatibility Laboratory Testing Requirements

HLA Typing

D1.000 HLA A, B, Bw4, Bw6, C, DR, and DQB antigens. When reporting DR antigens, DRB1 and DRB3/4/5 must be reported. The lab is encouraged to report splits for all loci as shown in Appendix 3A. Laboratories that perform deceased donor typing to be used in kidney, kidney-pancreas, pancreas, or pancreas islet allocation must report molecular typing results (at the level of serological splits) for all required antigens prior to organ offers.

D1.100 Laboratories performing HLA typing using cytotoxicity techniques must conform to all pertinent standards in Section H- Cytotoxicity Methods.

D1.200 Laboratories performing HLA typing using nucleic acid analysis must conform to all pertinent standards in Section K- Nucleic Acid Analysis.

D1.300 If alternative methods are used for HLA typing, procedures must be defined and validated, and must include sufficient controls to ensure accurate assignment of types. All relevant standards from the above sections must be applied.

D2.000 Typing Assignment

D2.100 Each HLA antigen must be defined by a sufficient number of reagents to clearly define each antigen or allele group for which the laboratory tests.

D2.200 The level of resolution of HLA typing must be appropriate for the clinical application.

D2.300 The method of assignment of HLA phenotypes must be documented for each technique used.

D2.400 The laboratory must have and adhere to a written policy that establishes when antigen redefinition and retyping are required.

D2.500 The laboratory must maintain a list of antigens and/or alleles defined by each test used in the laboratory.

D3.000 Reagent Validation

D3.100 Cell or DNA panels of known HLA class I and class II phenotype must be available to validate new typing reagents.

D3.200 The specificity of typing reagents obtained locally or from other sources and used for preparation of local trays must be documented and confirmed by external and/or internal QC testing.

D3.300 Each lot and/or shipment of new commercial reagents must be evaluated. The laboratory must establish and employ detailed policies and procedures for such evaluations

D3.400 Techniques used must be validated to optimally define HLA class I and/or class II antigens and/or alleles.

D4.000 HLA Typing by Nucleic Acid Analysis

D4.100 The HLA alleles detected by each primer, probe or template primer combination must be defined. Primers and probes must be tested with all alleles that are recognized by the W.H.O. Nomenclature Committee for Factors of the HLA System, provided that nucleotide sequences and reference DNA are readily available.

D4.200 The laboratory must have a process to recognize and document ambiguous combination(s) of alleles for each template/primer or probe combination.

D4.300 Typing by Sequenced Based Typing (SBT)

D4.310 Templates must have sufficient specificity for a locus or allele to provide interpretable primary sequencing data.

D4.320 Each unknown sequence must be compared with the sequences of all alleles that are recognized by the W.H.O. Nomenclature Committee for Factors of the HLA System provided that the nucleotide sequences are readily available.

D4.330 The laboratory must maintain records that define the sequence database utilized to interpret the primary data. This database must be updated at least annually. If a determined sequence is ambiguous (i.e., more than one possible interpretation of available data) the report must indicate all possible allele combinations.

E Antibody Screening

E1.000 Laboratories performing assays using cytotoxicity must conform to standards in Section H - Cytotoxicity Methods.

E1.100 Laboratories performing assays using flow cytometry based methods must conform to the standards in Section L1.000 Instrument Standardization/Calibration and in Section L2.000 Flow Cytometric Crossmatch Technique.

E1.200 Laboratories using ELISA techniques for antibody screening must conform to Standards in Section M- Enzyme Linked Immuno Sorbent Assay (ELISA).

E1.300 Laboratories using solid phase multichannel arrays for antibody screening must conform to Standards in Section N.

E2.000 Techniques

E2.100 The laboratory must determine the antibody specificities that can be identified by the technique(s) used. The technique(s) must be appropriate for the clinical application.

E2.200 To detect antibodies to HLA class II antigens, a method must be used that distinguishes them from antibodies to HLA class I antigens.

E2.300 There must be a procedure in place to monitor and adjust for non-specific binding of antibody.

E2.400 Appropriate methods or controls must be used to assess the impact of xenogeneic and/or monoclonal therapeutic antibodies.

E3.000 Sera

E3.100 Sera must be tested at a concentration(s) determined to be optimal for detection of antibody(ies) to HLA antigens. The dilution(s) must be documented in the test records.

E3.200 All tests must include an appropriate positive and negative control.

E4.000 Panel and Target Selection

E4.100 The panel of antigens must be sufficient in number and phenotypic distribution with respect to individual antigens and/or crossreactive groups (CREGs) for the population served and for the intended use of the test results.

E4.200 For assays intended to provide information on HLA antibody specificity, documentation of the HLA class I and/or class II phenotypes of the panel must be maintained.
E4.300 Target cells or purified HLA molecules must be appropriate. The concentration, condition and phenotype of target cells or purified HLA molecules must be sufficient to ensure the antibodies being tested for (either HLA class I or class II) can be detected.

F Renal and Pancreas Organ Transplantation

F1.000 If deceased donor transplants are performed, personnel for the required histocompatibility testing must be available 24 hours a day, seven days a week.

F2.000 HLA Typing

F2.100 Prospective typing of deceased donors for HLA-A, B, C, Bw4, and Bw6, and DR, DR51, DR52, DR53 and DQB antigens is mandatory.

F2.200 Prospective typing of candidates for HLA-A, B, Bw4, Bw6 and DR is mandatory, and the typing of C, DR51, DR52, DR53, and DQB is highly recommended.

F3.000 Antibody Screening

F3.100 Laboratories must have a policy in place to evaluate the extent of sensitization of each patient at the time of initial evaluation and following potentially sensitizing events, based on the antibody characteristics that are clinically relevant to each transplant center's protocols. The transplant program must provide this information to the laboratory.

F3.200 Laboratories must have a program to periodically screen serum samples from each patient for antibody to HLA antigens. The laboratory must have a documented policy establishing the frequency of screening serum samples and must have data to support this policy. Samples must be collected at time intervals outlined in the joint agreement between the laboratory and the transplant program.

F3.300 It is highly recommended that serum samples be tested for antibody to HLA antigens and that 1) information about antibody specificity be considered when evaluating the patient for transplant and, 2) that serum samples having defined class I and/or class II specificities be used in crossmatch testings.

F3.400 It is highly recommended that the HLA class I and class II specificity of antibodies be identified and reported and be distinguished from antibodies to non-HLA antigens.

F4.000 Crossmatching

F4.100 The laboratory must be capable of performing a prospective crossmatch and must do so when requested by a physician or other authorized individuals. Histocompatibility laboratories must have a joint written policy with their transplant program(s) on transplant candidate crossmatching strategies.

F4.200 Techniques

F4.210Although the laboratory may use the basic complement-dependent microlymphocytotoxicity test for determining donor-recipient compatibility, it must also use a crossmatching technique with increased sensitivity.

F4.220 Crossmatches must be performed with potential donor T lymphocytes. It is recommended that crossmatches be performed with B lymphocytes using a method that distinguishes between reactions with T and reactions with B lymphocytes.

F4.230 The laboratory must have and adhere to a written policy determining the serum(a) used in the final crossmatch. The relevance of the policy must be supported by published data or data generated in the laboratory. The policy must consider or include historic and current sensitizing events.

F4.300 Samples

F4.310 Sera must be tested at a dilution that is optimal for each assay.

F4.320 The laboratory must have a policy for storage and maintenance of recipient sera. The policy must define the samples to be retained and the duration of storage.

G Other Organ and Islet Cell Transplantation

G1.000 The laboratory must HLA type all potential transplant recipients and donors when requested by a physician or other authorized individuals.

G2.000 Patients must be screened for the presence of anti-HLA antibodies at initial evaluation and following sensitizing events when requested by a physician or other authorized individuals. It is recommended that unacceptable antigens be identified to optimize donor selection.

G3.000 The laboratory must be capable of performing a prospective crossmatch and must do so when requested by a physician or other authorized individuals.

G4.000 Histocompatibility laboratories must have a joint written policy with their transplant program(s) on transplant candidate antibody screening/identification and crossmatching strategies.

G5.000 Techniques with increased sensitivity in comparison with the basic/NIH complement-dependent micro-lymphocytotoxicity test must be used.

H1.000 Cytotoxicity Methods

1.000 For each cell-serum combination, the results must be recorded in a manner that indicates the approximate percent of cells killed.

1.100 Each laboratory must have a written policy that assigns positive or negative results based on percentage of cells killed.

H2.000 Controls

2.100 Each tray must include at least one positive control serum that reacts with all cells expressing the class of antigens being tested.

2.200 Each tray must include at least one negative control serum documented to be non-reactive under the specified test conditions.

2.300 Cell viability in the negative control well at the end of incubation must be sufficient to ensure accurate interpretation of results.

2.400 Appropriate methods or controls must be used to assess the impact of xenogeneic and/or monoclonal therapeutic antibodies in patient samples on the cytotoxicity assay.

H3.000 Target Cells

3.100 When testing enriched cell populations the level of purity must be sufficient to ensure accurate interpretation of results.

H4.000 Complement

H4.100 Each lot and/or shipment of complement must be tested to determine that it mediates cytotoxicity in the presence of specific antibody, but is not cytotoxic in the absence of specific antibody. Optimal performance must be established and documented.

H4.200 Complement must be tested separately for use with each type of target cell (i.e., T-cells, B-cells, CLL cells) and with each test method used, since a different dilution or preparation may be required for optimal performance.

I ABO Blood Group Determination

I1.000 Laboratories performing ABO blood group determination must use techniques compliant with Federal regulations.

I2.000 If testing for the A1 subgroup of ABO group A is performed, the extract of Dolichos biflorus must be used at a dilution and with a technique documented not to agglutinate non-A_1 cells. Each assay or batch test run must include known A1 and non-A_1 cells as controls.

I3.000 If titration of anti-ABO antibodies is performed, the procedure and criteria for interpretation must be established and validated by the laboratory.

I4.000 Laboratories using molecular techniques for ABO blood grouping must conform to all pertinent standards in Section K- Nucleic Acid Analysis.

J Chimerism Analysis

J1.000 Laboratories performing engraftment and chimerism testing using nucleic acid analysis must conform to all pertinent standards in Section K- Nucleic Acid Analysis.

J2.000 The specificity and sequence of primers must be defined. The genetic designation (e.g., locus) of the target amplified by each set of primers must be defined and documented. For each locus analyzed, the laboratory must have documentation that includes the chromosome location, the approximate number of known alleles, and the distinguishing characteristics (e.g., sizes, sequences) of the alleles that are amplified.

J3.000 If sample processing involves the isolation of cell subsets or specific hematopoietic cell lineages, the laboratory should document the purity obtained whenever possible. If purity is not documented for a given sample, then this information must be provided on the patient report.

J4.000 For each locus tested, patient and donor samples collected pre-transplant, and/or control samples demonstrated to have similar performance characteristics (e.g., sensitivity, competition in PCR) must be amplified and analyzed concurrently with patient samples collected post-transplant.

J5.000 Analysis and Reports

J5.100 Potential for preferential amplification of different sized alleles must be assessed and considered in the analysis.

J5.200 If more than one locus is amplified in a single amplification (multiplex), the effects of such amplification on each system must be assessed and considered in the analysis.

J5.300 Reports must identify the genetic loci analyzed according to standard nomenclature or published reference. For RFLP testing, the restriction endonuclease used and the fragment size must be identified.

J5.400 If results are reported in a quantitative or semi-quantitative manner, criteria for evaluating the relative amounts of recipient and donor in a mixed chimeric sample must be established.

J5.500 When mixed chimerism is not detected, reports must state the sensitivity level of the assay.

K Nucleic Acid Analysis

K1.000 Universal Standards (The standards in K1 apply to all nucleic acid testing).

K1.100 Nucleic Acid Extraction

K1.110 Nucleic acids must be purified by standard methods that have been validated in the laboratory and have written guidelines specifying the minimal acceptable sample (e.g., volume, number of cells, type of cells). If tests are performed without prior purification of nucleic acids, the method(s) used must specify the minimum acceptable sample and must fulfill all of the criteria set forth in A5.000.

K1.120 If nucleic acids are not used immediately after purification, samples must be stored under conditions that preserve their integrity.

K1.130 Nucleic acids must be of sufficient quality (e.g., purity, concentration) to ensure reliable test results.

K1.200 Electrophoresis

K1.210 Each electrophoretic run must include negative and positive controls that are processed with each assay to verify adequate and appropriate PCR amplification of target DNA.

K1.220 If size of the resulting nucleic acid fragment is a critical factor in the analysis of the data, the following steps must be undertaken: 1) the amount of DNA loaded in each lane must be within a range that ensures equivalent migration of DNA in all samples, including size markers, and 2) size markers that produce discrete electrophoretic bands spanning and flanking the entire range of expected fragment sizes must be included in each gel.

K1.230 The laboratory must establish criteria for accepting validity of each gel and of each lane of the gel and must determine and validate acceptable electrophoretic conditions for each assay.

K1.300 Analysis

K1.310 Acceptable limits of signal intensity must be specified for positive and negative results. If these are not achieved, corrective action is required.

K1.320 Two independent interpretations of primary data are required.

K1.330 Automated systems and computer programs must be validated prior to use and tested routinely for accuracy and reproducibility of manipulations.

K2.000 Template Amplification

K2.100 Facilities and Equipment

K2.110 Laboratories performing amplification of nucleic acids must establish and employ protocols to prevent DNA contamination using physical and/or biochemical barriers. Pre-amplification procedures must be performed in a work area that excludes amplified nucleic acid that has the potential to serve as a template in any amplification assays performed in the laboratory.

K2.120 The use of dedicated equipment and reagents as well as physical and/or biochemical barriers must be used to prevent nucleic acid contamination (carry-over).

K2.130 The laboratory must perform procedures to remove carry-over contamination from work areas used for manipulation of pre-amplification reagents or samples.

K2.140 When using methods that utilize two consecutive steps of amplification, addition of the template for subsequent amplifications must occur in an area isolated by physical or chemical barriers from both the pre-amplification work area and post-amplification work areas.

K2.150 Each work area (i.e., pre-amplification, secondary amplification, and post-amplification) must have dedicated pipettors. Positive displacement pipettes or filter-barrier tips are recommended for pre-amplification and secondary amplification work areas.

K2.160 Thermal cycling instruments must precisely and reproducibly maintain the appropriate temperature of samples. Accuracy of temperature control for samples must be verified at least every 6 months.

K2.170 Incubators and water baths must be monitored for accurate temperature maintenance every time the assay is performed.

K2.200 Reagents

K2.210 All reagents (solutions containing one or multiple components) utilized in the amplification assay must be dispensed in aliquots for single use or reagents can be dispensed in aliquots for multiple use if documented to be free of contamination at each use.

K2.220 Reagents used for initial amplification must not be exposed to post- amplification work areas. Reagents used for secondary amplification must be stored in an area that prevents carry-over contamination.

K2.300 Primers

K2.310 Primers must be stored under conditions that maintain specificity and sensitivity.

K2.320. Conditions that influence the specificity or quantity of amplified product must be demonstrated to be satisfactory for each set of primers.

K2.330 Laboratories must have a policy for quality control of each lot and shipment of primers using reference or well-characterized material.

K2.340 For labeled primers the specificity and robustness of the detection method must be validated. For those laboratories that store these reagents for extended periods, their performance must be periodically confirmed.

K2.400 Amplification Templates

K2.410 Samples containing nucleic acids that will be amplified (e.g., blood, DNA isolates) must be stored under conditions that do not result in artifacts, inhibition of the amplification reaction, and exposure to post-amplification work areas or any other sources of carry-over contamination.

K2.420. The acceptable range for the amount of target must be specified and validated.

K2.500 Contamination

K2.510 Nucleic acid contamination must be monitored for the most common amplification products that are produced in the laboratory. Routine wipe tests of pre-amplification work areas must be performed. Monitoring must be performed using a method that is at least as sensitive as routine test methods. If

amplified product is detected, the area must be cleaned to eliminate the contamination and retested. Corrective measures must be taken to prevent future contamination.

K2.520 At least one negative control (no nucleic acid) must be included in each amplification assay. Testing of open tubes in the work area is recommended.

K2.600 Controls and Quality Assurance

K2.610 The quantity of specific amplification products must be monitored (e.g., gel electrophoresis, hybridization).

K2.620 Criteria for accepting or rejecting an amplification assay must be specified.

K2.630 If presence of an amplified product is used as the end result, controls must be included to detect amplification in every amplification mixture. Amplification specificity must be monitored on a periodic basis.

K2.640 If an amplified product is used as a nucleic acid target, variation in the amount of amplified product must be monitored (e.g., hybridization with a consensus probe, gel electrophoresis). The acceptable range for the amount of test DNA must be specified.

K3.000 Technique-Specific Standards

K3.100 Oligonucleotide Probe Assays

K3.110 The specificity and target sequence of oligonucleotide probes must be defined.

K3.120 Oligonucleotide probes must be stored under conditions that maintain specificity and sensitivity.

K3.130 Oligonucleotide probes must be utilized under empirically determined conditions that achieve the defined specificity. Laboratories must perform quality control testing to confirm specificity for each lot and shipment of probe. Reference material must be used for quality control whenever possible.

K3.140 Oligonucleotide probe specificity and detection method sensitivity must be established and must be documented to be reproducible before results are reported.

K3.150 Hybridization must be carried out under empirically determined conditions that achieve the defined specificity.

K3.160 The laboratory must have a validated procedure for reuse of nucleic acids (probes or targets) bound to solid supports or in solution. Controls must be included to ensure sensitivity and specificity of the assays are unaltered.

K3.200 Sequence Specific Amplification

K3.210 Each amplification reaction must include procedures to detect technical failures (e.g., an internal control such as additional primers or templates that produce a product that can be distinguished from the typing product).

K3.300 Other Techniques

K3.310 All methods must be validated in the laboratory, as described under A5.000.

K3.320 Appropriate controls must be included for each component of the test.

L Flow Cytometry

L1.000 Instrument Standardization/Calibration

L1.100 An optical standard, consisting of latex beads or other uniform particles, must be run to ensure proper focusing and alignment of all lenses in the path for both the exciting light source and signal (light scatter, fluorescence, etc.) detectors.

L1.110 Standard(s) must be run for each fluorochrome used to ensure adequate amplification of the fluorescent signal(s). These fluorescent standards may be incorporated in the beads or other particles used for optical standardization (ref. L1.100) or may be a separate bead or fixed cell preparation.

L1.120 Both the optical and fluorescent standards must be run each time the instrument is turned on and any time maintenance, adjustments, or problems have occurred during operation that could potentially affect instrument function.

L1.130 The results of optical focusing/ alignment must be recorded in a daily quality control log.

L1.140 Threshold values for acceptable optical and fluorescent standardization results must be established for all relevant signals for each instrument used.

L1.150 In the event a particular threshold value cannot be attained, there must be a written protocol detailing the corrective action required.

L1.200 If performing analyses that require the simultaneous use of two or more fluorochromes, an appropriate procedure must be used to compensate for overlap in their emission spectra.

L1.300 Laser power output and current input (amps) must be recorded daily for each instrument. Acceptable thresholds and corrective action protocols must be documented.

L2.000 Flow Cytometric Crossmatch Technique

L2.100 The laboratory must ensure the appropriate definition and purity of cell populations by the use of either a multi-color technique or other documented method.

L2.110 The laboratory must assess the binding of human immunoglobulin using a fluorochrome labeled reagent such as either an F(ab')2 anti-human IgG that is specific for the Fc region of the heavy chain or other documented method.

L2.120 Crossmatch results for a specific cell population (e.g., T-cells, B-cells and/or monocytes) must be based on the use of a monoclonal antibody that detects an appropriate cluster designated antigen (e.g., CD3 for T cells, CD19 or CD20 for B cells and CD14 for monocytes).

L2.130 Each laboratory must establish and document the optimum serum/cell ratio.

L2.200 Controls

L2.210 The negative control must be human serum documented to be non-reactive against the crossmatch target cells.

L2.220 The positive control must be human antibody of the appropriate isotype for the assays and specific for the antigens that are targeted in the crossmatch. Positive controls must be used at a dilution appropriate for the assay (i.e., a dilution at which moderate changes in assay sensitivity are likely to be detected) and must react with appropriate target cells from all humans.

L2.230 The anti-human immunoglobulin reagent must be titered to determine the dilution with optimal activity (signal to noise ratio). If a multicolor technique is employed, the reagent must not demonstrate crossreactivity with the other immunoglobulin reagents used to label the cells.

L2.240 Regardless of the method used for reporting raw data (mean, median, mode channel shifts or quantitative fluorescence measurements), each lab must establish its own threshold for discriminating positive reactions. Any significant change in protocol, reagents, or instrumentation requires repeat determination of the positive threshold.

L2.300 Interpretation

L2.310 Each laboratory must define the criteria used to define positive and negative crossmatches.

L2.320 Appropriate methods or controls must be used to assess the impact of xenogeneic and/or monoclonal therapeutic antibodies on flow crossmatches.

L3.000 Immunophenotyping By Flow Cytometry

L3.100 Terminology used must conform to the most recent publication of the International Workshop of Differentiation Antigens of Human Leucocytes or other appropriate scientific organizations.

L3.200 Cell Preparation

L3.210 The method used for cell preparation must be documented to yield appropriate preparations of viable cells sufficient to ensure accurate test results.

L3.220 For internal labeling, the method used to allow fluorochrome labeled antibodies to penetrate the cell membrane must be documented to be effective.

L3.300 Quality Control

L3.310 Specificity controls, consisting of appropriate cell types known to be positive for selected standard antibodies must be run within laboratory-defined intervals sufficiently short to assure the proper performance of reagents.

L3.320 A negative reagent control(s) must be identified for each test cell preparation. It is recommended that this control consist of monoclonal antibody(ies) of the same species and subclass and be prepared/purified in the same way as the monoclonal(s) used for phenotyping.

L3.330 For indirect labeling, it is recommended that the negative control reagent be an irrelevant primary antibody and the same secondary antibody(ies) conjugated with the same fluorochrome(s) used.

L3.340 For direct labeling, it is recommended that the negative control reagent be an irrelevant antibody conjugated with the same fluorochrome and at the same fluorochrome: protein ratio used in all relevant test combinations.

L3.350 Each laboratory must define acceptable time periods between processing, labeling and analysis of samples. Control samples must be treated in the same manner.

L3.360 Gating strategies must be employed to assure that the population of interest is being selected without significant contamination.

L3.370 Conclusions about abnormal proportions or abnormal numbers of cells bearing particular internal or cell surface markers must only be drawn in comparison with local `control' data obtained with the same instrument, reagents and techniques.

L3.380 Determination of percent positives must take into consideration the results of the negative control reagent.

L3.400 Reagents

L3.410 The laboratory must have a policy to validate the specificity of monoclonal antibodies, either by using appropriate controls or by testing in parallel with previous lots.

L3.420 The quantities of reagents used for each test sample must be determined by the manufacturers or from published data and whenever possible be verified locally by titration.

L3.430 Monoclonal antibodies that have been reconstituted from lyophilized powder form for storage at 4^oC must be processed according to the manufacturer's instructions or locally documented procedures to remove microaggregates prior to use in preparation of working stains.

M Enzyme Linked Immuno Sorbent Assay (ELISA)

M1.000 Instrument Standardization/Calibration

M1.100 The ELISA Reader

M1.110 The light source and filter must produce the intensity and wavelength of light required for the test system.

M1.120 Calibration/verification of plate alignment and instrument linearity must be performed according to the manufacturer's instructions or at least once every 6 months and must be documented.

M1.200 If used, microplate washer performance must be checked monthly and acceptable performance must be documented.

M2.000 ELISA Technique

M2.100 Each assay must contain positive , negative and reagent controls that are appropriate for the intended use of the assay and the test results. The dilution of reagents and test specimens must be documented.

M2.200 For an assay to be valid it must be documented that all controls meet or exceed established thresholds as specified in the assay procedure.

M2.300 Sample identity and proper plate orientation must be maintained throughout the procedure.

N Solid Phase Multi-channel Arrays

N1.100 Instrument Standardization/Calibration

N1.100 Instruments must be standardized and/or calibrated as described under the relevant sections of L1.000 Flow Cytometry: Instrument Standardization/Calibration.

N1.200 Calibration/verification of plate alignment and instrument linearity must be performed according to the manufacturer's instructions or at least once every 6 months and the precise movement of the tray/plate must be documented.

N1.300 If used, microplate washer performance must be checked monthly and acceptable performance must be documented.

N2.000 Reagents

N2.100 Assays must use positive, negative and reagent controls that are appropriate for the intended use of the assay and the test results. Any dilution or optimization of reagents and/or test specimens must be documented.

N2.200 For an assay to be valid it must be documented that all controls meet or exceed established thresholds specified in the assay procedure.

N3.000 Testing

N3.100 Sample identity and proper plate orientation must be maintained throughout the procedure.

N3.200 PRA Determination

N3.210 The quality control of the new system's reagents must adhere to the standards described in M3.300, M3.400, and N2.100.

N3.300 Histocompatibility Typing

N3.310 If the typing system is probe based, all standards relating to SSO procedures (Section K3.100) are applicable and must be adhered to.

D.3. PRESERVATION OF ZERO MISMATCH TISSUE TYPING MATERIALS

For future studies of HLA identification, tissues suitable for the isolation of DNA or purified DNA itself, from both the organ donor and recipient, should be preserved for each 0 mismatched cadaveric kidney transplant. If tissue is preserved it should be preserved by the recipient transplant center HLA laboratory, under conditions which maintain the integrity of the DNA, for at least 5 years. This rule is applicable only when biologic specimens in excess of that necessary for the performance of required biologic tests are available.