

MICROAMS[®]

Micro-Antibody Monitoring System

INTENDED USE

MICROAMS[®] is a qualitative solid phase enzyme linked immunosorbent assay (ELISA) designed to detect IgG antibodies to donor-specific HLA class I and class II glycoproteins.

For *In Vitro* Diagnostic Use.

SUMMARY AND EXPLANATION

HLA is a major antigenic system in determining the survival of transplant allografts or transfused platelets in sensitized individuals.¹ HLA antibodies can be acquired through alloimmunization as a result of pregnancy, transfusion of blood products, or previous transplants. In general, alloimmunization leads to the production of HLA antibodies in approximately 33% of exposed individuals.² HLA class I and class II molecules are polymorphic membrane glycoproteins. Antibodies to HLA glycoproteins have been shown to contraindicate successful transplantation.³⁻⁶ Post-transplant monitoring of these antibodies is important in assessing potential graft survival.^{7,8}

PRINCIPLES OF THE PROCEDURE

HLA glycoproteins are prepared from donor lymphocytes by solubilizing the cells with a non-ionic detergent. Once solubilized, the lymphocyte lysates are added to microwells to which monoclonal antibodies specific for HLA class I or class II have been immobilized. HLA glycoproteins are allowed to bind to the monoclonal antibodies, and unbound glycoproteins are washed away. The microwells containing the bound glycoproteins are tested with human serum to detect antibodies against HLA molecules. Unbound antibodies are then washed away. An alkaline phosphatase labeled anti-human globulin reagent (Anti-IgG) is added to the wells and incubated. The unbound Anti-IgG is washed away and the substrate PNPP (p-nitrophenyl phosphate) is added. After a 30-minute incubation period, the reaction is stopped by a sodium hydroxide solution. The optical density of the color that develops is measured in a spectrophotometer.

This kit employs three control systems:

The first system is comprised of a dried lymphocyte (Dried Lymphocyte Control) and an antibody (Positive Serum Control) known to be reactive with both the class I and class II glycoproteins on the lymphocyte. The dried lymphocyte is rehydrated and solubilized to create a lysate. The lysate is applied to the reagent control wells. The Positive Serum Control is tested against control lysate to confirm that the Lymphocyte Lysis Buffer and other assay reagents are working properly.

The second control system is comprised of two alkaline phosphatase conjugated monoclonal antibodies. One (Class I Lysate Control Reagent) is directed against HLA class I glycoproteins. The other (Class II Lysate Control Reagent) is directed against HLA class II glycoproteins. When tested directly against the captured glycoproteins in the wells, these reagents will give positive results, showing that HLA class I or HLA class II molecules have been captured in the wells.

The third control system is the negative control. Positive reactions are denoted by test wells that are greater than two times (2x) the average value of the negative control wells.

REAGENTS

Maximum number of tests per kit: 44

All reagents should be stored as directed by the label.

- | | | |
|------------|----|---|
| MS1 | 1. | Microwells: Low volume, flat bottom microwell strips to which monoclonal antibodies specific for HLA class I (color-coded blue) or HLA class II (color-coded purple) have been immobilized. Ready for use. The |
| MS2 | 2. | microwell strips are enclosed in resealable foil pouches. |
| TCW | 3. | Concentrated Wash (10x): Tris (hydroxymethyl) aminomethane buffered solution containing sodium chloride, Tween 20, and 1% sodium azide. Dilute with deionized or distilled water before use. Store Working Wash solution up to 48 hours at room temperature (22 to 25°C) or up to seven days at 2 to 8°C. |
| SD | 4. | Specimen Diluent: Phosphate buffered saline solution containing bovine albumin, mouse serum, and 0.1% sodium azide. Ready for use. |

- | | |
|--------------|--|
| SB | 5. Substrate Buffer: This solution contains diethanolamine, magnesium chloride, and 0.02% sodium azide. Ready for use. Protect from light. |
| SS | 6. Stopping Solution: 3M Sodium Hydroxide. Ready for use. Use with care. |
| AG | 7. Conjugate (100x): Alkaline phosphatase conjugated goat anti-human immunoglobulin G (IgG) and 0.1% sodium azide. (Color-coded white cap.) Dilute in Lysate and Conjugate Diluent before use. |
| LCD | 8. Lysate and Conjugate Diluent: Tris buffered solution containing sodium chloride and 0.05% sodium azide. (Color-coded blue cap.) Ready for use. |
| LLB | 9. Lymphocyte Lysis Buffer (10x): Tris buffered saline containing a non-ionic detergent and 0.5% sodium azide. Dilute in deionized or distilled water before use. |
| LCRI | 10. Class I Lysate Control Reagent (LCRI) (100x): Alkaline phosphatase conjugated mouse anti-human immunoglobulin G (IgG) specific for HLA class I glycoprotein and 0.1% sodium azide. (Color-coded blue cap.) Dilute in Lysate and Conjugate Diluent before use. |
| LCRII | 11. Class II Lysate Control Reagent (LCRII) (100x): Alkaline phosphatase conjugated mouse anti-human immunoglobulin G (IgG) specific for HLA class II glycoprotein and 0.1% sodium azide. (Color-coded purple cap.) Dilute in Lysate and Conjugate Diluent before use. |
| DLC | 12. Dried Lymphocyte Control Pellet: Dried lymphocytes. Prepare as directed. |
| PN | 13. PNPP (p-nitrophenyl phosphate) Substrate: Crystalline powder. Reconstitute with deionized or distilled water and dilute in Substrate Buffer before use. Protect from light. |
| PC | 14. Positive Serum Control (4x): Human Serum. 0.1% sodium azide. Dilute in Specimen Diluent before use. |
| NC | 15. Negative Serum Control (4x): Human Serum. 0.1% sodium azide. Dilute in Specimen Diluent before use. |
| PS | 16. Plate Sealers. |

PRECAUTION

- Do not use reagents that are turbid or contaminated.
- Do not use reagents beyond their expiration date.
- Substitution of components other than those provided in this kit may lead to inconsistent or erroneous results, however any lot of GTI Lymphocyte Lysis Buffer can be used to make lysates.
- Microwells and reagents contained in the kit are not to be used in conjunction with any other test system.
- When making dilutions, follow pipette manufacturer's instructions for appropriate dispensing and rinsing techniques.
- Accurately calibrated pipets should be used for reagent additions.
- Clumping of the lymphocytes prior to lysing can result in poor lysate preparations and result in low values when tested with the Lysate Control Reagents. It is important to treat the cells gently to avoid clumping. Contamination of a lymphocyte preparation with red cells may lower the quantity of HLA glycoproteins in the lysate if not removed prior to estimation of packed cell volume.
- Centrifuging at higher than recommended speeds may cause premature lymphocyte lysis, clumping, and loss of lymphocytes.
- Lysates may be stored frozen at -70 to -80°C before use. Lysates should not be refrozen.
- Care **MUST** be taken to avoid contamination of Lysate and Conjugate Diluent, LCRI, LCRII, and the Conjugate. Inadvertent contamination of these reagents with human serum will result in the neutralization of the LCRI, LCRII, and Conjugate and subsequently to test failure.
- Discard any unused portions of diluted Conjugate, diluted Lymphocyte Lysis Buffer, diluted Lysate Control Reagents, diluted Positive and Negative Control, and diluted and reconstituted PNPP reagent after each assay.
- The enzyme substrate reaction occurring in the final incubation is temperature sensitive and should be performed in a controlled area at 22 to 25°C.
- Due to variations in instruments or consistently higher or lower room temperatures, it may be necessary for the laboratory to establish a slightly longer or shorter incubation time in order to consistently achieve valid control results. Because the

temperature of the final incubation can affect control values, it is important to periodically monitor the room temperature incubation.

- Due to the small size of the low volume microwells, it is essential that the plate reader be checked for proper alignment.
- Do not use a reference wavelength when reading the plate in the plate reader.

CAUTION

- All human serum used in the Positive and Negative Controls for this product has been tested and found negative for antibody to HIV, HCV and HBsAg by FDA approved methods. No test method, however, can offer complete assurance that HIV, Hepatitis C virus, Hepatitis B virus or other infectious agents are absent. Therefore, these materials should be handled as potentially infectious.
- Some of the reagents supplied with this kit contain sodium azide as a preservative.
WARNING: Sodium azide reacts with lead and copper plumbing forming highly explosive metal azides. When discarded in a sink, the sink should be flushed with a large volume of water to prevent azide buildup. Sodium azide is a poison and is toxic if ingested.
- Stopping Solution (NaOH) is corrosive. Avoid contact with skin and eyes. Spills should be cleaned up immediately.
- Discard all components when completed according to local regulations.

SPECIMEN COLLECTION

Cells: Spleen should be harvested according to local procurement procedures. Spleen should be processed within 72 hours of harvesting. Peripheral blood lymphocytes should be collected in sodium heparin or ACD tubes using aseptic technique, stored at room temperature, and should be processed within 72 hours of collection.

Serum: Blood should be collected without anticoagulant (serum) using aseptic technique.

Serum samples should be tested while still fresh to minimize the chance of obtaining false positive or false negative reactions due to improper storage or contamination of the specimen.

Serum samples that cannot be tested immediately should be stored at 2 to 8°C for no longer than 48 hours or frozen. Samples frozen at -20°C or below remain in good condition for 2 years. However, in order to avoid the deleterious effect of repeated freeze/thaw cycles, it is recommended that samples be aliquoted in small volumes and then stored frozen. Avoid storage in a frost-free freezer.

Serum should be separated from red cells when stored or shipped.

Particulates or aggregates in the serum sample can cause false positive reactions or poor duplicate values. Samples containing particulate matter should be clarified by centrifugation prior to testing.

Only whole human serum is suitable for this assay. Prior dilution of samples in anything other than human serum which does not contain antibodies to HLA glycoproteins could affect the results.

Microbially contaminated, hemolyzed, lipemic, icteric, or heat inactivated sera may give inconsistent results and should be avoided.

PROCEDURE

Materials Provided:

Vials may contain more reagent than described on the labels. Be sure to measure the reagent with an appropriate device when making dilutions.

1. 12 – 1 x 8 low volume microwell strips with holder containing immobilized monoclonal antibody to HLA class I glycoproteins with a blue stripe
2. 12 – 1 x 8 low volume microwell strips with holder containing immobilized monoclonal antibody to HLA class II glycoproteins with a purple stripe
3. 1 x 125 mL Concentrated Wash
4. 1 x 14 mL Specimen Diluent
5. 1 x 14 mL Substrate Buffer
6. 1 x 14 mL Stopping Solution
7. 1 x 120 µL Anti-Human IgG Conjugate
8. 1 x 14 mL Lysate and Conjugate Diluent
9. 1 x 2.5 mL Lymphocyte Lysis Buffer
10. 1 x 14 µL Class I Lysate Control Reagent
11. 1 x 14 µL Class II Lysate Control Reagent

12. 1 x pellet, Dried Lymphocyte Control Pellet
13. 6 x 50 mg PNPP Substrate
14. 1 x 200 μ L Positive Serum Control
15. 1 x 200 μ L Negative Serum Control
16. 18 Plate Sealers

Additional Materials Required:

1. Test tubes for patient sample and control dilutions and for reagent dilutions
2. Transfer pipets
3. Adjustable micropipets to deliver 1 – 10 μ L, 10 – 100 μ L and 100 – 1,000 μ L and disposable tips
4. Timer
5. Microplate reader capable of measuring OD at 405 or 410
6. Deionized or distilled water
7. Absorbent paper towels
8. Microplate washer or device
9. Centrifuge
10. 37°C incubator or waterbath
11. Cell culture media
12. Lymphocyte density separation media (1.077 g/mL)
13. Ice

This procedure is divided into three main parts: “Dried Lymphocyte Control Lysate Preparation”, “Donor Lysate Preparation”, and “Test Procedure”. Helpful Hints:

1. Donor and control lysate may be prepared ahead of time and aliquots may be frozen at -70 to -80°C. If donor and control lysate have been previously prepared, go to “Test Procedure”.
2. For each donor lysate used in the assay, a Negative Control and a Lysate Control must be included.
3. The Reagent Control (Positive Serum Control) must be included in each class I and class II assay.
4. To reduce the detrimental effect of proteases on the HLA glycoproteins, the lymphocyte lysates can be held on ice for a short period of time not exceeding 4 hours.
5. Promptly freeze any remaining undiluted donor or lymphocyte control lysate in small aliquots (single use) in sealed and labeled tubes at -70 to -80°C for up to two years.
6. It is critical to spin at the recommended rcf. DO NOT vary the established centrifugation rate.

Dried Lymphocyte Control Lysate Preparation:

1. Rehydrate Dried Lymphocyte Control
 - a) Rehydrate the Dried Lymphocyte Control Pellet (**DLC**) by adding 500 μ L of cell culture media to the dried pellet of cells.
 - b) Allow to stand at room temperature for at least 1 hour.
 - c) Dislodge the button of cells with the aid of a pipet tip and vortex to obtain a cell suspension.
 - d) Centrifuge the mixture at 1,000 to 1,500 rcf for 5 minutes to pellet the cells.
 - e) Leave the supernatant on the cells until cells are to be lysed.
2. Lysis of Dried Lymphocyte Control
 - a) Prepare 0.5 mL of diluted lysis buffer by adding 50 μ L of Lysis Buffer to 450 μ L of deionized or distilled water. Store diluted lysis buffer on ice for up to 4 hours.

NOTE: Lymphocyte Lysis Buffer is viscous. Prime the pipet tip 2-3 times in Lymphocyte Lysis Buffer before dispensing and rinse after addition to deionized or distilled water. Mix well.

- b) Remove the supernatant from the rehydrated Dried Lymphocyte Control (**DLC**).
- c) The rehydrated Dried Lymphocyte Control (**DLC**) should be lysed in 500 μ L of *diluted* Lymphocyte Lysis Buffer. Mix well with the aid of a pipet tip and by vortexing to completely resuspend the cells. Centrifuge the mixture at 1,000 to 1,500 rcf for 3 – 5 minutes to sediment the cell membranes. Transfer supernatant into a clean labeled tube.
- d) The Dried Lymphocyte Control Lysate should be kept on ice until it is used in the assay (not to exceed 4 hours). The Dried Lymphocyte Control Lysate can be stored frozen at -70 to -80°C for future use.

Donor Lysate Preparation:

1. Calculating amount of cells to prepare

- a) Assign a location for each serum sample to be tested using the enclosed Recording Sheets.
- b) Complete the first calculation on the Lysate Calculation Worksheet to determine the amount of *diluted* lysate needed.
- c) Complete the second calculation to determine the volume of *undiluted* lysate needed for the class I strips.
- d) Complete the third calculation to determine the volume of *undiluted* lysate needed for the class II strips.
- e) For the fourth calculation, add the volume of class I lysate to the volume of class II lysate to obtain the total volume of undiluted lysate required.
- f) Use the last calculation to determine the volume of cells to prepare.
- g) Document all dilutions and calculations on the worksheet.

2. Isolating Donor Cells

Lymphocytes may be harvested from either whole blood or spleen. Whole blood or spleen should be processed within 72 hours of harvesting.

Whole Blood:

- a) If using whole blood, layer on a lymphocyte density separation media (1.077 g/mL) and centrifuge at 1,000 to 1,500 rcf for 15 to 20 minutes. Collect the layer of cells at the density separation media interface. Care should be taken to minimize the number of red cells in the preparation.
- b) Transfer the suspension to a large tube and centrifuge at 1,000 to 1,500 rcf for 5 to 10 minutes to pellet the cells. Remove the supernatant.
- c) Add a volume of cell culture media equal to at least 5 times the volume of the pellet. Gently resuspend the cells.
- d) Centrifuge to pellet the cells and remove supernatant (first wash).
- e) Repeat c) and d) for a total of 3 washes.
- f) After final wash leave supernatant on cells until cells are to be lysed.

Spleen:

- a) To prepare lymphocytes from spleen, macerate to break up tissue into small particles, suspend in complete cell culture media, layer on lymphocyte density separation media (1.077 g/mL) and centrifuge at 1,000 to 1,500 rcf for 15 to 20 minutes. Collect the layer of cells at the density separation media interface.
- b) Transfer the suspension to a large tube and centrifuge at 1,000 to 1,500 rcf for 5 to 10 minutes to pellet the cells. Remove the supernatant.
- c) Add a volume of cell culture media equal to at least 5 times the volume of the pellet. Gently resuspend the cells.
- d) Centrifuge to pellet the cells and remove supernatant (first wash).
- e) Repeat c) and d) for a total of 3 washes.
- f) After final wash, leave supernatant on cells until cells are to be lysed.

3. Lysis of Donor Cell

- a) For every 100 μ L of packed lymphocytes to be lysed, prepare 1.0 mL of diluted lysis buffer by adding 100 μ L of Lysis Buffer to 900 μ L of deionized or distilled water. Store diluted lysis buffer on ice for up to 4 hours.

NOTE: Lymphocyte Lysis Buffer is viscous. Prime the pipet tip 2-3 times in Lymphocyte Lysis Buffer before dispensing and rinse after addition to deionized or distilled water. Mix well.

- b) Transfer the required volume of donor cells into a test tube by either of the following two methods:
 - i)
 - Remove the supernatant from the pellet of washed cells.
 - The packed cell volume can be verified by adding an equal volume of water to an identical test tube and measuring the volume of water.
 - Add an equal volume of cell culture media to the button of cells to make a 50% cell suspension.
 - Mix well and transfer twice the volume of packed cells needed (calculated in step 1 above) to a test tube.
 - Centrifuge the cell suspension at 1,000 to 1,500 rcf for 5 to 10 minutes to obtain a pellet containing the desired volume of lymphocytes.
 - Remove the supernatant.
- c) For every 10 μ L of packed donor cells ($\approx 30 \times 10^6$ cells) add 100 μ L of *diluted* Lymphocyte Lysis Buffer.

Mix well with the aid of a pipet tip and by vortexing to completely resuspend the cells. Centrifuge the mixture at 1,000 to 1,500 rcf for 3 – 5 minutes to sediment the cell membranes. Transfer supernatant into a clean labeled tube.

- ii) Alternatively, count the lymphocytes and transfer (using the following table as a guide) the appropriate number of cells into a polypropylene test tube. Centrifuge at 1,000 to 1,500 rcf for 5 minutes to obtain a pellet of lymphocytes. Remove the supernatant.

| Volume of Packed Lymphocytes | Estimated # Lymphocytes Obtained from Peripheral blood | Estimated # Lymphocytes Obtained from Spleen cells |
|------------------------------|--|--|
| 10 μ L | 30 x 10 ⁶ cells | 30 x 10 ⁶ cells |
| 20 μ L | 50 x 10 ⁶ cells | 60 x 10 ⁶ cells |

If frozen lysate is being used, thaw an aliquot just prior to use and dilute appropriately as described in step 4 of Test Procedure (see below).

For Class I strips, 2 μ L of undiluted lysate or 6 x 10⁵ lysed cells are needed per well.

For Class II strips, 4 μ L of undiluted lysate or 12 x 10⁵ lysed cells are needed per well.

Test Procedure

- Bring all reagents to room temperature (22 to 25°C).
- Make Working Wash solution by diluting Concentrated Wash (**TCW**). Add 1 volume of Concentrated Wash to 9 volumes of deionized or distilled water. Mix well.
- Determine the number of patient samples to be tested.
- Dilute an appropriate amount of donor or Dried Lymphocyte Control lysate in Lysate and Conjugate Diluent (**LCD**) according to the calculations on the work sheet as follows:
 - Label one tube “Donor Class I Lysate”. Prepare appropriate amount of lysate for class I strips by diluting 1 part lysate with 7 parts Lysate and Conjugate Diluent (**LCD**).
 - Label a second tube “Donor Class II Lysate”. Prepare appropriate amount of lysate class II strips by diluting the 1 part lysate with 3 parts Lysate and Conjugate Diluent (**LCD**).
 - Label a third tube “Dried Lymphocyte Control”. Prepare appropriate amount of dried lymphocyte control lysate by diluting 1 part dried lymphocyte control lysate with 3 parts Lysate and Conjugate Diluent (**LCD**). Please note that the dilution for the dried lymphocyte control is the same for both class I and class II strips and that this control should be used in the reagent control wells on both type of strips.
- Remove the microwell frames (MS1 or MS2) from pouch. Remove the required number of MS1 and MS2 strips. Promptly reseal unneeded strips in the protective pouch.

NOTE: Only two frames are provided in the kit. Do not discard until all strips have been used.

NOTE: Orient the frame with A1 in the top left corner. Be sure that all strips are properly seated and snapped into their frame. Label or number each strip to avoid errors. Maintain the same plate orientation throughout the assay.

- Add 15 μ L of *diluted* Dried Lymphocyte Control lysate to the wells of both class I and class II strips designated as Reagent Control.
- Add 15 μ L of *diluted* class I or class II donor lysate to all assigned patient, Negative Control, and Lysate Control wells on each class I (blue) and class II (purple) strip.

NOTE: If multiple patient samples are tested at the same time, LABEL EACH STRIP TO AVOID ERRORS.

NOTE: Do not add lysate, samples or reagents to blank wells.

Be sure to add the diluted class I lysate to the blue strips and the diluted class II lysate to the purple strips. Adding the wrong dilution may result in failure of the assay.

- Seal the microwells with a plate sealer and incubate for 30 minutes in a 37°C waterbath. If a dry incubator is used instead, increase time by 10 minutes.

PREPARE SAMPLES AND CONTROLS

9. Dilute the Positive Serum Control (**PC**), Negative Serum Control (**NC**), and patient serum in Specimen Diluent (**SD**) as follows and mix well.

| | Volume Specimen Diluent (SD) | Volume sample |
|-----------------------------------|---------------------------------------|---------------|
| PC | 60 µL | 20 µL |
| NC (per donor lysate) | 60 µL | 20 µL |
| Patient Sample (per donor lysate) | 60 µL | 20 µL |

NOTE: This will provide enough samples to test two class I wells and two class II wells. Centrifuge patient sera at high speed in a microcentrifuge for 5-10 min. Remove the supernatant for use.

10. WASH STEP:

- Aspirate or decant contents of each well and blot on absorbent toweling.
- Add 140 µL Working Wash solution.
- Aspirate or decant.
- Repeat steps b + c for a total of 3 washes.
- Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

11. Using the Recording Sheet as a guide, add diluted patient samples and control reagents to the assigned wells as follows:

- Add 15 µL of diluted Positive Serum Control to the Reagent Control wells of both strips containing Dried Lymphocyte Control lysate.
- Add 15 µL of diluted Negative Serum Control to Negative Control wells.
- Add 15 µL of Lysate and Conjugate Diluent to the Lysate Control wells of the class I and the class II strips. This will prevent them from drying out during the next incubation period.
- Add 15 µL of diluted patient serum sample to designated wells containing donor lysate.

12. Seal the microwells with a plate sealer and incubate for 30 minutes in a 37°C waterbath. If a dry incubator is used instead, increase time by 10 minutes.

NOTE: During the incubation period perform the following two steps:

13. Dilute 1 part Conjugate (**AG**) with 100 parts Lysate and Conjugate Diluent (**LCD**). Use a polypropylene container.

| Strips: | 4 - 1x8 | 24 - 1x8 |
|---------|---------|----------|
| AG | 7 µL | 50 µL |
| LCD | 700 µL | 5 mL |

NOTE: Conjugate is viscous. Prime the pipet tip 2-3 times in Conjugate before dispensing and rinse tip after addition to Lysate and Conjugate Diluent. Mix well.

14. Dilute the Lysate Control Reagents (**LCRI** and **LCRII**) in Lysate and Conjugate Diluent (**LCD**) as follows:

| | |
|---------------|--------|
| LCRI or LCRII | 2 µL |
| LCD | 198 µL |

Mix Well.

NOTE: Accurate measurement of the LCR reagents is important in obtaining acceptable Lysate Control values.

15. WASH STEP:

- Aspirate or decant contents of each well and blot on absorbent toweling.
- Add 140 µL Working Wash solution.
- Aspirate or decant.
- Repeat steps b + c for a total of 3 washes.
- Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

NOTE: It is important to completely remove all wash solution after the final wash.

16. Add 15 µL per well of the Lysate Control Reagents to the appropriate Lysate Control wells:
 - a) add diluted LCRI to the class I strips (blue)
 - b) add diluted LCRII to the class II strips (purple).
17. Add 15 µL per well of *diluted* Conjugate (prepared in a previous step above) to all remaining wells except the Lysate Control and Blank wells.
18. Seal the microwells with a plate sealer and incubate for 30 minutes in a 37°C waterbath. If a dry incubator is used instead, increase time by 10 minutes.

NOTE: During the incubation period, perform the following two steps:

17. Dissolve PNPP Substrate (**PN**) by adding 0.5 mL deionized or distilled water to the vial. Replace stopper and mix well. Protect from light until use.
18. Dilute the PNPP (**PN**) with the Substrate Buffer (**SB**) as follows:

| Strips: | 4 - 1x8 | 24 - 1x8 |
|---------|---------|----------|
| PN | 20 µL | 120 µL |
| SB | 2 mL | 12 mL |

Mix well. Protect from light until use. Can be stored up to 45 minutes at room temperature (22 to 25°C) before use.

21. WASH STEP:

- a) Aspirate or decant contents of each well and blot on absorbent toweling.
- b) Add 140 µL Working Wash solution.
- c) Aspirate or decant.
- d) Repeat steps b + c for a total of 3 washes.
- e) Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

Proceed promptly through next three steps.

22. Add 50 µL of diluted PNPP solution to all wells EXCEPT those designated as BLANKS.
23. Allow the microwells to stand in the dark for 30 minutes at ROOM TEMPERATURE (22 to 25°C).

NOTE: Incubation time and temperature after the addition of PNPP is critical. DO NOT vary the established incubation time or temperature. For consistency, begin timing promptly after addition of the reagent to the first well.

24. Stop the reaction by adding 50 µL of Stopping Solution (**SS**) to each well in the same sequence as the addition of substrate. Add 100 µL of Stopping Solution to the blank wells.
25. Read the absorbance (OD) of each well at 405 or 410 nm. If the results cannot be read immediately, return the wells to a dark location for up to 30 minutes.

NOTE: Do not use a reference wavelength when reading the plate on the plate reader.

26. Subtract the values obtained in the blank wells from all sample and control wells. Many ELISA readers are programmed to automatically perform this step.
27. Record the results on the Recording Sheet.

QUALITY CONTROL

Quality control of MICROAMS[®] is built into the test system by the inclusion of Positive and Negative Serum Controls and the Lysate Control Reagents. These controls should be included with each test run to help determine if technical errors or reagent failures have occurred.

Criteria for a valid test:

| | | |
|---------|-----------------------|----------------------|
| | Negative Control (NC) | Reagent Control (PC) |
| Mean OD | ≤ 0.300 | ≥ 1.000 |

The Lysate Control Reagent wells must demonstrate positive reactivity (>2x the value obtained for the mean of the negative control). A positive reaction in the Lysate Control wells indicate that HLA glycoproteins are captured in those wells. This control will not necessarily identify an improperly prepared or diluted lysate, or assure suitable serological reactivity of the glycoprotein.

In a study using 35 different spleen derived lysates typical results for class I Lysate Control wells had an OD range of 1.255 – 3.049 with a mean OD value of 2.124 and for class II Lysate Control wells had an OD range of 0.766 – 2.366 with a mean OD value of 1.808.

OD readings for positive patient samples obtained from duplicate tests should fall within 20% of the mean of the duplicates. Samples whose results are outside of this limit should be retested.

NOTE: Poor duplicates can be the result of reagent or sample omission, uneven addition or mixing of reagents, poor washing techniques, uneven temperature during incubations, misalignment of the plate reader, stray light during the final incubation, or cross-well contamination. Failure to test in duplicate may lead to acceptance of erroneous results.

INTERPRETATION OF RESULTS

Test results showing OD values greater than twice (2x) the value obtained for the mean of the negative controls are regarded as positive results.

LIMITATIONS

Erroneous results can occur from bacterial contamination of test materials, inadequate incubation periods, inadequate washing or decanting of test wells, exposure of substrate to stray light, omission of test reagents, exposure to higher or lower than prescribed temperature requirements, insufficient or excessive lysate or omission of steps.

The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause an increased non-specific binding and produce false-positives in this assay.

The results of this assay should not be used as the sole basis for a clinical decision.

Some low titer, low avidity antibodies may not be detected using this assay.

This assay will detect IgG antibodies only.

The performance characteristics of this product were established using sera containing antibodies with known reactivities against the HLA A, B, and DR loci.

SPECIFIC PERFORMANCE CHARACTERISTICS

To ensure suitable reactivity and specificity, each lot of MICROAMS[®]: HLA Class I and Class II is tested prior to release with samples known to contain donor specific HLA antibodies.

Performance Evaluation

MICROAMS[®] was tested against the GTI Antibody Monitoring System (AMS[®]) kit in a method comparison study. A total of 101 samples were evaluated on MICROAMS[®] and AMS[®] for reactivity to Class I HLA. A total of 101 samples were evaluated on MICROAMS[®] and AMS[®] for reactivity to Class II HLA.

| | | | | |
|-------------------------------|----------|--------------------------|----------|-------|
| | | AMS [®] Class I | | |
| | | Positive | Negative | Total |
| MICROAMS [®] Class I | Positive | 52 | 0 | 52 |
| | Negative | 0 | 49 | 49 |
| | Total | 52 | 49 | 101 |

Agreement: 100%

Comparative Method: GTI Antibody Monitoring System: HLA Class I and Class II (AMS[®]1+2)

| Class I | Co-positivity/Sensitivity | Co-negativity/Specificity |
|------------------------------|---------------------------|---------------------------|
| Value | 100% | 100% |
| 95% confidence level (lower) | 93.1% | 92.7% |
| 95% confidence level (upper) | 100% | 100% |

| | | AMS [®] Class II | | Total |
|--------------------------------|----------|---------------------------|----------|-------|
| | | Positive | Negative | |
| MICROAMS [®] Class II | Positive | 49 | 0 | 49 |
| | Negative | 0 | 52 | 52 |
| | Total | 49 | 52 | 101 |

Agreement: 100%
 Comparative Method: GTI Antibody Monitoring System: HLA Class I and Class II (AMS[®]1+2)

| Class II | Co-positivity/Sensitivity | Co-negativity/Specificity |
|------------------------------|---------------------------|---------------------------|
| Value | 100% | 100% |
| 95% confidence level (lower) | 92.7% | 93.1% |
| 95% confidence level (upper) | 100% | 100% |

Precision

To evaluate MICROAMS[®] precision, inter-assay and intra-assay studies were conducted. In each case 2 known positive and 2 known negative samples were tested against 2 different lysates. Evaluation of all data was done according to NCCLS User Protocol for Evaluation of Qualitative Test Performance: Approved Guideline EP12-A. The data demonstrated 100% agreement within-run (n=20), 100% agreement between runs (n=40), and 100% agreement lot-to-lot (3 lots, n=24) in the reportable results obtained.

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