Chapter: Enzyme Linked Immunosorbent Assay (ELISA)

Section: Application module

Module : Antibody Detection

Author(s): Pat Adams, Ohio State University: adams-5@medctr.osu.edu
Dean Sylvaria, Beth Israel Deaconess Medical Center: dsylvari@bidmc.harvard.edu
Kathryn Tinckam, University Health Network - Toronto: Kathryn.tinckam@uhn.on.ca

Date Prepared: 10/08/2005

OBJECTIVES

- Understand and identify the components of the ELISA antibody identification assay including the target antigens, secondary antibodies, enzyme substrates and their by-products.

- Identify a positive or negative reaction, understand how this determination was obtained, and how it applies to the clinical assessment of the transplant candidate.

- Understand and identify the ASHI standards that pertain to the compliance of the assay, the plate reader and automated washer, and the quality control measures incorporated in the test system.

INTRODUCTION

The ELISA (enzyme-linked immunosorbent assay) assay is designed to detect antibodies that bind to antigens coated onto solid surfaces. One application of this assay is the qualitative screening for the presence or absence of anti-MHC Class I or Class II antibodies.

Enzyme-conjugated antibodies are used to detect the binding of IgG to antigens coupled to the plastic surface of a microwell. The antigens coated to the plastic surface of the microwell have been obtained from EBV transformed B-cell lines from among a large pool of donors. These antigens have been tested against known anti-sera to ensure reactivity against a broad range of known HLA specificities. Secondary antibodies (e.g. goat anti-human IgG) are often linked to enzymes such as horse radish peroxidase or alkaline phosphatase. These enzymes are coupled to the Fc region of the IgG molecule, leaving the Fab regions free to bind to their specific antigen, human IgG. A colorless enzyme substrate is used to detect the binding of the secondary antibodies, which, if present, convert the substrate into a colored enzyme by-product. Thus, the production of a colored by-product, greater than two times the average of the negative controls, in the ELISA antibody identification assay indicates the presence of MHC-reactive alloantibodies in the serum of a transplant candidate.

The presence of MHC-reactive alloantibodies indicates the need for further testing of the transplant candidate's antibody profile. Once it has been determined that an anti-HLA IgG antibody is present, the next step is to attempt to determine the specificity of the antibody detected. If no antibody is detected, the transplant candidate is considered unsensitized and no further antibody testing is required at this time. Candidates who are considered sensitized should be tested monthly while unsensitized candidates may be tested on a quarterly basis unless a sensitizing event, such as a blood transfusion, has been documented. In such a case, the candidate should have a two week post transfusion sample tested and, subsequently, be tested for three consecutive months if no reactivity is detected.

ELISA testing is sensitive, versatile, and most importantly, specific for the detection of antibody binding to purified MHC proteins. Simply put, alloantibody testing by ELISA replaces the

complex and dynamic cell surface used in cell panel-based testing, with a solid matrix (plastic well), coated with a single class of protein (MHC Class I or MHC Class II) for the purpose of detecting alloantibody.

While the simplification of the ligand target (plastic surface v cell surface) is undoubtedly the priority advantage, ELISA testing offers several additional advantages to cell-surface based testing. There are no cell viability issues. The reaction score (optical density) is objective, and not dependent on subjective technologist interpretation. ELISA is also an ideal approach to testing serial samples, and can be very useful in monitoring patients undergoing antibody reduction protocols. Similarly, it is an easy way to monitor alloantibody production during the post transplant period.

METHODOLOGIES

Commercial kits are available and come supplied with all required reagents, as well as, having undergone extensive quality control measures to determine the appropriate working conditions of the assay. The manufacturer provides training for their product and continuous technical support. Validation of the assay is required by the laboratory prior to clinical use, regardless of the quality control provided by the manufacturer.

Alternately, the detection of anti-MHC Class I and Class II antibodies by ELISA can be developed in the laboratory, however, this requires extensive quality control and expertise.

PROTOCOLS

Procedure: Detection of Class I or II IgG HLA Antibodies by Enzyme-Linked Immunosorbent Assay (ELISA)

Purpose:

To describe the steps involved in testing patient sera for IgG antibodies directed against HLA Class I or II antigens. Controls and patient sera are added to separate wells containing previously bound affinity purified HLA Class I or II glycoproteins obtained from EBV transformed B lymphocytes. Unbound antibody is washed away and an anti-human IgG antibody is allowed to attach to the HLA antibody present. Any unattached IgG is washed away and a chromogenic substrate added. The intensity of color in each well is read by an ELISA reader. A negative cut-off is established using two times the mean of the negative control wells. Positive results are identified as an ocular density value greater than the negative cut-off. The information obtained from this assay will be used to identify potential recipients with antibody to HLA Class I or II antigens, to identify potential recipients who may require further identification of the Class I or II antibody detected, and to establish crossmatch requirements for some potential recipients.

Materials:

GTI™ Class I-Screen or B-Screen Kit
- 12 ELISA Microwell Strips 1 X 8 Wells, with Holder
- 1 X 60 ml Concentrated Wash Solution
- 1 X 15 ml Specimen Diluent Solution
- 1 X 80 ul Anti-Human IgG Reagent
- 6 X 500 ul PNPP (p-nitrophenyl phosphate) Reagent
- 1 X 15 ml Enzyme Substrate Buffer
- 1 X 15 ml ELISA Stopping Solution (3M Sodium Hydroxide Solution)
- 1 X 450 ul Positive Serum Control
- 1 X 0.7 ml Negative Serum Control
- 12 Plate Sealers

Microcentrifuge tubes
Micro-Centifuge
Deionized or distilled water
Transfer pipets
Micropipets (10-100 ul & 100-1000 ul)
Pipet tips
Microplate reader

Procedure:

1. Bring all reagents to room temperature.

2. Make working wash solution using a 1:10 dilution of the Concentrated Wash Solution with deionized or distilled water.

3. Assign and record each patient sample two wells on the recording sheet.

4. If using removable strips, remove required strips and reseal unused strips in pouch provided. Strips placed in frame must have yellow line at the bottom of the strip. If a complete tray is to be used, remove it now.

5. Dilute positive and negative controls and patient sera 1:2 using Specimen Diluent Solution. Mix well.

6. Add 250 ul of Working Wash Solution to all wells. Let stand for 5-10 minutes at room temperature.

7. Decant all wells by flipping contents out, inverting tray onto absorbent material and tapping against a solid surface making sure to blot any residual fluid.

8. Add 50 ul of diluted Negative Serum Control to designated wells according to position on the recording sheet.

9. Add 50 ul of diluted Positive Serum Control to designated wells according to position on recording sheet.

10. Add 50 ul of each diluted patient serum to designated wells according to position on recording sheet.

11. Cover plate with plate sealer and incubate in dry incubator at 37^{o}C for 45 minutes.

12. Decant contents of each well by flipping contents out, inverting tray onto absorbent material and tapping against a solid surface making sure to blot any residual fluid. Add 200-300 ul of Working Wash Solution to each well. Repeat wash step for a total of four washes. Keep trays inverted on moist paper towel.

13. Dilute anti-human IgG 1:1000 in Specimen Diluent. Mix well.

14. Add 50 ul diluted IgG to all wells except those identified as Blanks. Cover plate with plate sealer and incubate in dry incubator at 37^{o}C for 45 minutes.

15. Decant contents of each well by flipping contents out, inverting tray onto absorbent material and tapping against a solid surface making sure to blot any residual fluid. Add 200-300 ul of Working Wash Solution to each well. Repeat wash step for a total of four washes. Keep trays inverted on moist paper towel. Proceed quickly through the next three steps.

16. Dissolve one vial of PNPP Substrate with 500 ul of deionized or distilled water.

17. Dilute PNPP Substrate 1:1000 in Enzyme Substrate Buffer. Mix well avoiding direct light.

18. Add 100 ul of diluted PNPP solution to all wells except those identified as Blanks. Incubate in the dark at 22-25^{o}C for exactly 30 minutes (do not seal plate).

19. Add 100 ul of ELISA Stopping Solution to all wells. Add an additional 100 ul of ELISA Stopping Solution to wells designated as Blanks.

20. Read the absorbance of each well at 405 nm within 15 minutes of adding the stop solution. Allow at least five minutes before reading plate (keep in dark until read and analyzed). Be sure to align the plate with the Blank wells in the lower left corner when loading into the ELISA reader.

21. Print ocular density readings of each well and transcribe the results to appropriate wells on the recording sheet. Calculate the average of the two readings for each well.

22. Calculate the mean value of negative control wells. The mean value must be between 0.100 and 0.250 to be valid. The positive control must have an ocular density greater than or equal to 1.500.

23. Establish a negative cut-off value, which is equal to two times the mean of the negative controls. Record this value on the B-Screen Recording Sheet.

24. Identify sera which have an average ocular density reading greater than or equal to the negative cut-off. These sera are positive for the detection of antibody to HLA Class II antigens.

Limitations:

1. Each assay detects only the Class of IgG HLA antibodies identified by the manufacturer (I or II) and, therefore, will not detect other HLA antibodies, IgM antibodies or any other class of immunoglobulin, or non-HLA antibodies. Some non-cytotoxic HLA antibodies may be detected that are not observed in the standard lymphocytotoxic assay.

2. This assay is not intended to identify an antibody but rather to detect the presence or absence of an antibody. Antibodies with low titer or low avidity to HLA Class I or II may not be detectable.

3. Antibody to low frequency HLA Class I or II antigens may not be detected by this assay.

4. Immune complexes or aggregates may cause non-specific binding leading to false positive reactions.

5. Anti-coagulated samples and samples treated with immunoglobulin therapies, such as IVIG and ATG, may have false positive or false negative results.

6. The ocular density value for the positive control must be equal to or greater than 1.500 and the mean of the negative controls must be between 0.100 and 0.250.

Precautions:

This assay involves the handling of potentially infectious serum and appropriate precautions should be taken when handling patient and control serum. Some reagents contained in the kits contain sodium azide as a preservative. Sodium azide reacts with lead and copper plumbing forming highly explosive metal azides. If flushed down a sink, reagents containing sodium azide should be flushed with large amounts of water to prevent azide build up.

This procedure is a modification of the manufacturer's product insert guidelines.

QUALITY CONTROL/ASSURANCE

Procedure: Quality Control for ELISA Class II Antibody Screen and Identification

Purpose:

To describe the standards involved in maintaining appropriate quality control measures to ensure the validity and accuracy of the ELISA Class II screen and identification methodology. The quality control of these assays is dependent on both mechanical performance and biological reagents. Some of the quality control standards are accounted for in daily laboratory QC. Some standards are performed at longer intervals, i.e. pipet calibration, and others are needed every time the assay is run. Daily quality control records are documented on the ELISA Class II Quality Control Checklist and stored in the ELISA Class II Daily Quality Control Log.

Materials:

Refrigerator (0-10°C)
Dry Incubators (22°C and 37°C)
Microcentrifuge
Micropipets (10-100 ul & 100-1000 ul)
Microplate reader
Commercial ELISA kits

Standards:

1. Ensure refrigerator holding screen and identification kits are within acceptable temperature range. Monitored daily with results written in Daily QC book.

2. Ensure 22°C and 37°C incubators are within acceptable temperature range. Monitored daily with results written in Daily QC book.

3. Check documentation of microcentrifuge for verification of performance standards. Calibration of microcentrifuge performed by Wilkinson Associates, Inc.. Documentation can be found on sticker on the side of the microcentrifuge.

4. Verify calibration of micropipets (refer to notebook for pipetman/Hamilton calibration). Micropipets will be designated for ELISA use only. Calibration to be performed bi-annually.

5. Check that microplate reader is enabled and communication with computer is available. Turn power switch on rear of reader to “on” position and start-up computer. Open software program on computer hard disk. Under [Control] on the menu bar select “Close Drawer”, then select “Open Drawer.” Verify that the microplate reader performs a self-calibration.

6. Run Linear Validation Plate (LVP) once a month to check photo diode linearity of the reader. Results of the LVP are to be stored in a notebook labeled Photo Diode Linearity Notebook.

7. Reagents should be examined for expiration dates. Do not use reagents which have exceeded the manufacturer’s expiration date. All reagents should be visually examined for turbidity or contamination. Discard any reagents found to be turbid or contaminated.

8. Internal quality control measures in the form of positive and negative controls have been incorporated into each assay. Verify that Positive and Negative Control sera are added to each microplate to be tested and results are within acceptable limits.

Corrective Action:

Deviation from an acceptable range or specifications in any of the quality control standards for ELISA Class I or II screening or identification assays must be resolved immediately. Resolution may include but is not limited to: discarding reagents, mechanical repairs (performed by authorized service representatives), repeating assays or performing confirmatory testing of the assay using well-characterized anti-sera. Any corrective action must be documented.

ELISA Class II Quality Control Checklist

GTI™ B-Screen & Quik-ID Class II

Technologist:_____

Date: _____

PASS/FAIL	OBJECTIVE
_____	Emax precision microplate reader communicating with computer.
_____	Emax precision microplate reader operates self-calibration.
_____	MedTec Linear Validation Plate (LVP) run to check photo diode linearity. Results documented if applicable (monthly).
_____	Reagents examined for expiration dates - documented on worksheets.
_____	Reagents examined for turbidity or contamination.
_____	Positive and Negative Control sera plated and results within acceptable limits. B-Screen: O.D. for Positive Control must be = 1.500 Mean O.D.of Negative Controls must be 0.100-0.250 Quik-ID: Mean O.D. of Positive Contols must be = 8X the mean of the Negative Controls.

Corrective Action (if necessary): _____

_____

_____

FREQUENTLY ASKED QUESTIONS

Question: Can this assay detect antibodies other than those classified as IgG?

Answer: Yes, if patient serum is suspected to have anti-HLA antibodies other than of the Class IgG, replacing the IgG secondary antibody with an IgM class antibody will allow for detection of IgM anti-HLA class antibodies. The secondary anti-human IgM antibody will attach to the HLA antibody present in the assay. A chromogenic substrate is added and the color change measured and quantified. A secondary IgM Class antibody is not included in commercially available kits.

Question: Is the established negative cut-off an absolute criteria for identification of a positive or negative determination?

Answer: No, the negative cut-off is a quantitative measurement that establishes a threshold for what can be defined as either positive or negative, but is not absolute. There is a range above and below this negative cut-off, which must be determined by each laboratory individually, that should indicate repeat or reflex testing by the same or a more sensitive method.

Question: Why are blank wells included in the assay and what function do they serve?

Answer: Blank wells are included as part of the assays internal control system. Plastics have an inherent ability to absorb light at different wavelengths. The absorbance, measured in units of ocular density, detected in these blank wells is subtracted from the ocular density values obtained in the patient and control wells. In this way, the absorbance used to determine the positivity of a serum sample does not include any inconsequential absorbance due to the nature of the plastic material used to make the microwell.

FREQUENTLY ASKED QUESTIONS

Question: Can a percent reactive antibody or PRA be determined by the ELISA antibody identification assay?

Answer: No, this assay is not quantitative. It simply identifies the presence or absence of anti-MHC antibodies. There is data that suggests that the positivity of a reaction in units of ocular density may correlate with a quantitative value, but to date, this has not been established.

STANDARDS

D.4.6.13 Laboratories performing ELISA must ensure that components of the test system are prepared according to the manufacturer's directions and verify their performance. If the test system is modified, the laboratory must establish and verify the performance specifications.

D.4.3 Laboratories performing antibody screening and identification must have protocols that are appropriate for the clinical application.

D.4.6.18.1 Use a technique(s)that detects HLA-specific antibody with sensitivity equivalent or superior to that of the basic complement-dependent microlympho-cytotoxicity assay.

D.4.6.18.2 Use a panel of antigens sufficient in number and phenotypic distribution with respect to individual antigens and/or crossreactive groups (CREGs)for the intended use of the test results and for the population served.

D.4.6.18.10 Document that the pooled cells or antigens, used for a present/ not present detection of antibody, include the major antigen specificities or CREGs or be derived from a population of sufficient size to ensure representation of major antigen specificities.

D.4.6.18.11 Have a process for distinguishing HLA class I and class II antibodies from non-HLA antibodies as appropriate for clinical applications.

D.4.6.18.12 Ensure that there is a procedure to monitor and adjust for non-specific binding of antibody.

D.4.6.18.13 Use an HLA antibody screening method that is at least as sensitive or equivalent to, and predictive of the routine crossmatch method, and is consistent with clinical transplant protocols.

E3.100 Test sera at concentrations determined to be optimal for detection of antibody to HLA antigens and document the dilution(s)in the test records.

E3.200 Use a human serum demonstrated to be non-reactive as a negative control in each assay.

E3.200 Use a human serum of an appropriate isotype and specificity as a positive control in each assay.

E3.200 Use HLA molecules that are bound to solid supports (e.g., beads, plates)or expressed on human cells (e.g., peripheral blood, lymph node, spleen, cell lines)as targets.

E4.10 Use a panel of antigens sufficient in number and phenotypic distribution with respect to individual antigens and/or crossreactive groups (CREGs)for the intended use of the test results and for the population served.

E4.200 When applicable, use a method that detects antibodies to HLA class II antigens and distinguishes them from antibodies to HLA class I antigens.

E4.200 Document the HLA class I and/or class II phenotypes of the panel for assays intended to provide information on HLA antibody specificity.

D.4.11.20 Laboratories performing ELISA assays must use positive, negative, and reagent controls that are appropriate for the intended use of the assay and test results. The dilution of reagents and test specimens must be documented.

D.4.7.9 Laboratories performing ELISA techniques must follow the manufacturer's recommendations for verifying calibration and setting positive and negative cutoff values.

D.4.9.4.1.1 Demonstrate that the light source and filter produce the intensity and wavelength of light required for the test system.

D.4.9.4.1.2 Perform and document calibration/verification of plate alignment and instrument linearity according to the manufacturer's instructions (or at least once every six months).

D.4.9.4.2 Verify and record precise movement of the plate.

D.4.9.4.3 (Microplate Washer) Check and document microplate washer performance during each month of use.

LITERATURE CITED/RECOMMENDED READING

References:

GTI™ B-Screen product insert. 2001.

Dombrausky Osowski L: Quality assurance of information/data in the laboratory: New test validation, patient test management, computerization, and laboratory data maintenance. ASHI Laboratory Manual 4th Edition, Volume 1. 2000.

Dombrausky Osowski L, Gutierrez M, Muth B: HLA antibody screening and identification by ELISA methodology. ASHI Laboratory Manual 4th Edition, Volume 1. 2000.

Zachary AA: Solid Phase Immunoassays, tests for HLA-specific antibodies. ASHI NE Regional Ed, 1998.

Molecular Devices Corporation. Emax™ Precision Microplate Reader User's Manual. 1989.

Recommended Reading:

Lucas DP, Paparounis ML, Myers L, Hart JM, Zachary AA: Detection of HLA class I-specific antibodies by the QuikScreen enzyme-linked immunosorbant assay. Clin. Diag. Lab. Immunolol. 4: 252-257, 1997.

Zaer F, Metz S, Scornik JC: Antibody screening by enzyme-linked immunosorbant assay using pooled soluble HLA in renal transplant candidates. Transplantation 63: 48-51, 1997.

Bryan CF, Baier KA, Flora-Ginter G, Shield CF, Warady BA, et al.: Detection of HLA IgG antibodies by two enzyme-linked immunoassays, solubilized HLA class I and PRA-STAT: Comparison with the AHG PRA. Transplantation 60: 1588-1594, 1995.

Buelow R, Chiang T-R, Monteiro F, Cornejo MC, Ellingston L, et al.: Soluble HLA antigens and ELISA – a new technology for crossmatch testing. Transplantation 60: 1594-99, 1995.