Laboratory Procedure Manual

Analyte: Allergen-Specific IgE system

Matrix: Serum

Method: AlaSTAT Microplate Allergen-Specific IgE system

Allergen-Specific IgE in Serum – NHANES

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Many allergies are mediated by immunoglobulins of the IgE class. In sensitized individuals suffering from this immediate (atopic or anaphylactic) type of allergy, IgE molecules act as points of contact between the allergen and specialized cells that release histamine and other agents upon exposure to the allergen; this initiates the events which we recognize as allergic reactions.[5,10] When evaluated in the light of other clinical and laboratory findings, in vitro allergen-specific IgE tests can help the physician identify the allergen (or allergens) to which an individual is sensitive.

The AlaSTAT Microplate Allergen-Specific IgE system is an enzyme-labeled immunometric assay, based on liquid allergen complexes, monoclonal antibodies, and separation by ligand-coated wells. It represents a significant advance over conventional methods relying on allergens attached to a solid-phase support, such as a paper disk. Allergens, in a liquid format, are covalently bound to a soluble polymer/copolymer matrix, which in turn is labeled with a ligand – the same ligand used for coating the reaction wells. The use of an amino acid copolymer amplifies the amount of allergen that the matrix can support.

AlaSTAT Microplate assays use patented technology (U.S. Patent No. 4,778,751) exploiting liquid-phase kinetics in a microplate format. Ligand-labeled allergen complexes and a patient sample are pipetted into ligand-coated wells and then incubated for 1 hour. During this time, any endogenous IgE specific for the test allergen binds to it. Addition of a multivalent anti-ligand creates a bridge between the allergen/IgE complexes and the ligand-coated wells during the second 1-hour incubation. Separation of bound from free is then a simple matter of decanting and washing. The allergen/IgE complexes thus linked to the microplate wells are reacted with horseradish peroxidase-labeled monoclonal anti-IgE during a third 1-hour incubation, after which excess enzyme label is washed away. A chromogenic indicator (3, 3′,5,5′-tetramethylbenzidine) in a buffered hydrogen peroxide solution, reactive with the enzyme label, is then added, and the rate of color development is ascertained by monitoring the product using a kinetic microplate analyzer during a 5-minute read at 650 nm. Reaction rates, measured in milliOptical Density units per minute (mOD/min), are directly related to allergen-specific IgE concentrations. The reader makes the OD readings available to the WINMAX Windows software, which calculates the mOD/min, plots the calibration curve derived from 6 IgE calibrators, and calculates results for control and patient samples. The AlaSTAT system yields results both in familiar Class numbers and in a continuous concentration scale.

Sensitivity: Sensitivity benefits as a result of the high IgE-binding capacity of the AlaSTAT system, and also because the soluble matrix is able to support allergens which are carbohydrates, in addition to proteins and nucleic acids.[11]

Specificity: The system is not subject to interference from high total IgE levels and other nonspecific binding problems which affect solid-phase systems.[12]

2. SAFETY PRECAUTIONS

Observe Universal Precautions. Wear gloves, lab coat, and safety glasses at all times. Treat all specimens as potentially positive for HIV, Hepatitis B, and other blood borne pathogens. The AlaSTAT kit for performing Specific Allergen-IgE contains components of human origin which, when tested by FDA-approved methods, were found to be nonreactive for hepatitis B surface antigen and for HIV antibody. No known tests can guarantee, however, that products derived from human blood will not be infectious. Therefore, all components are handled as if capable of transmitting infectious agents. Sodium azide, at concentrations of less than 0.1 g/dL, has been added to certain components as a preservative. On disposal, flush with large volumes of water to prevent buildup of explosive metal azides in lead and copper plumbing. Decontaminate occasionally with 10% sodium hydroxide. Since peroxides are strong oxidizing agents, avoid all bodily contact with the TMB Substrate Solution. Dispose of leftover reagents as hazardous wastes. All leftover serum specimens and materials that have been in contact with blood products must be autoclaved before disposal.

3. COMPUTERIZATION: DATA SYSTEM MANAGEMENT

a. WinMAX software serves to collect and process AlaSTAT Microplate Allergen-Specific IgE data. The IgE concentrations of calibrators, controls, and patient samples are automatically calculated and printed with a Class number for each allergen tested. After the data are calculated and the final values are approved for release by the reviewing technologist, the results are transcribed by a technologist into the NHANES IV database which is located in an EXCEL file; data entry is proofed by the supervisor and a technologist. After each NHANES stand is completed, the supervisor will transmit the IgE values to the Westat Inc. mainframe computer along with other NHANES 99+ data.

b. Files stored on the network are automatically backed up nightly to tape by HIP support staff.

c. Documentation for data system maintenance is contained in hard copies of data records, as well as in "system log" files on the local hard drives used for archival of data.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special dietary instructions are given to donor.

b. Specimens for AlaSTAT Microplate Allergen-Specific IgE should be fresh or frozen serum. Collect blood by venipuncture[13] into plain tubes, avoiding hemolysis and separate the serum from the cells. Heparinized and EDTA plasma may also be used.

c. Specimens may be stored frozen at - 20°C in plastic vials for up to 6 months [16] or refrigerated at 2-8°C for 3 days.[14,16] Avoid repeated thawing and freezing. Do not attempt to thaw frozen specimens by heating them in a waterbath.

d. The procedure calls for 50 μL per well, and tests are run in duplicate

e. Samples that are partially clotted may not give accurate test results. Specimens are examined for clots through the use of wooden applicator sticks.

f. Neither bilirubin nor hemolysis has any clinically significant effect on the assay.

g. The use of an ultracentrifuge is recommended to clear lipemic samples.

h. Specimens should arrive frozen. Each specimen is labeled with a 9 digit specimen id. A paper manifest listing information about the specimens accompanies each shipment.

i. The day that the shipment is sent, a file, which is comma delimited, is sent via email. It contains the following information: specimen id, container slot number, date of collection, and the comment code associated with each specimen.

j. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies available in the NHANES laboratory and in the Special Activities Branch Specimen Handling Office). Specimen collection and transport and special equipment required are discussed in the protocol. In general, whole blood specimens should be transported and stored at no more than 4-8°C. Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen at ≤ -20°C.

5. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. Instrumentation

(1) DPC-MARK5 Robotic Pipettor is a standalone sample preparation unit. Samples can be aspirated from a tube rack or microplate, have up to three reagents added, be diluted, transferred and dispensed for reaction. Through the built-in software programs, a wide range of sample handling procedures can be performed automatically, rapidly and accurately.

(2) MAX 002 is an automated assay processor that can perform incubation, reagent addition, washing and reading of up to 6 plates without user intervention.

b. Materials

(1) 1000 mL volumetric cylinder

(2) Applicator sticks

(3) 10% bleach solution

(4) 70% isopropyl alcohol

(5) 1.8 mL polypropylene vials with screw caps

(6) Uncoated, 96 well microtiter plates

(7) 1- Liter plastic storage container for Delta Clean solution

(8) 1-Liter plastic storage container for Buffered Wash Solution

(9) 1-liter volumetric flask

(10) 500-mL square polypropylene container for blue dye solution

(11) Blue Dye Solution (Diagnostic Products Corporation, Los Angeles, CA)

(12) Buffered Wash Solution Concentrate (Diagnostic Products Corp.)

(13) Delta Clean (Diagnostic Products Corp.)

(14) Ligand-Coated Microplates (Diagnostic Products Corp.)

(15) Ligand-Labeled Anti-IgE Ab (Diagnostic Products Corp.)

(16) Enzyme-Labeled Anti-IgE Ab (Diagnostic Products Corp.)

(17) Anti-Ligand (Diagnostic Products Corp.)

(18) TMB Substrate Solution (Diagnostic Products Corp.)

(19) Standard Calibrator Module (Diagnostic Products Corp.)

(20) k82 latex allergen (Diagnostic Products Corp.)

(21) d2 Dermatophagoides farinae allergen (Diagnostic Products Corp.)

(22) Alastat Microplate Positive Control (Diagnostic Products Corp.)

(23) Alastat Microplate Negative Control (Diagnostic Products Corp.)

(24) Reverse osmosis water, greater than or equal to 10 megaOhm-cm at 25° C (supplied in-house)

c. Reagent Preparation

(1) Buffered wash solution concentrate (for washing microplate)[1]
Dilute 85 mL 10X buffered saline solution containing surfactants and preservative with 765 mL reverse osmosis water in a 1-L cylinder.

(2) Delta Clean alkaline detergent concentrate (for cleaning pipetting mechanisms)
Dilute 50 mL concentrated Delta Clean to volume with reverse osmosis water in a 1-liter volumetric flask.

(3) Blue Dye (for Precision Test)

Dilute with reverse osmosis water to give an approximate optical density reading of 1.0 when read with a 650nm filter with the MAX 002 spectrophotometer.

d. Standards (Standard Calibrator Module)

One set of six vials, labeled A through F, each containing 1.8 mL, with preservative. The calibrators contain the following amounts of IgE in kiloUnits per liter (kU/L0:

Calibrator A	0.35 kU/L
Calibrator B	0.7 kU/L
Calibrator C	3.5 kU/L
Calibrator D	17.5 kU/L
Calibrator E	52.5 kU/L
Calibrator F	100 kU/L

Stable at 2-8 degrees C for 30 days after opening or until the expiration date marked on the label.

6. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURE

(a) Standards contain 1.8 mL of liquid reagent, A through F, ready to use.

(b) Standards are transferred automatically to the ligand-coated microplate using the Mark 5 Robotic Pipettor as described in the procedure section of this method. The Max 002 is programmed to read the six standards in duplicate starting with level A.

(c) When all the standards have been read, the AlaSTAT Data Reduction program will prompt the printer to draw the linear standard curve (x = concentration, y = intensity). The generated curve is acceptable if the mean value of each standard, A through F, increases in value, if the percent CV of each standard, A through F, is less than 15%, and if the mean positive control value is greater than 1.4 and negative control values are less than 0.35.

(d) The AlaSTAT calibrators are standardized to the World Health Organization's Second International Reference Preparation for human serum IgE, number 75/502 (WHO 2nd IRP 75/502). Thus results from different labs can be compared.

7. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Preliminaries

(1) Thaw specimens of frozen serum at room temperature.

(2) Once per week the Mark 5 Soak Procedure is performed to clean the pipetter lines and probes. Using program 0, 3, 3, 3, the probes are soaked in 10% bleach solution and then in 70% isopropyl alcohol. Extended prime sequences are then performed using Reverse osmosis water once, Delta Clean once, and then Reverse osmosis water three more times using the program 0, 1, 3, 3for each extended prime sequence.

(3) Once per week run the Mark 5 Precision Test to validate that the instrument is pipetting consistently and precisely by using program 0, 7, 3, 3. The Mark 5 pipettes 25 uL of a Blue Dye solution into an uncoated 96 well microplate. OD reading is made on each well using the MAX 002 reader at a wavelength of 650 nm. The printer must be connected to print Precision Test results. Using the QUADCAL statistical value analysis package the mean, standard deviation, and coefficient of variation for the 96 well microplate is determined. The coefficient

of variation must be below 4.5% to be acceptable. If the CV is higher than this, the Mark 5 soak procedure is repeated, extended prime sequences are performed with Delta Clean and Reverse osmosis water, and the Mark 5 Precision test is repeated until values are within the acceptable range.

(4) Remove all test reagents from refrigerator (4° C) 30 minutes before use to allow them to equilibrate to room temperature (15-28° C).

(5) Make a worklist of the samples to be run under Worklist using the WinMAX program, and specify that they are to be analyzed for Specific IgE antibody.

b. Sample Preparation and Mark 5 formatting

(1) Check each specimen for clots with an applicator stick. Specimen must be free of fibrin clots. Then mix sample by inverting the capped vial gently several times.

(2) Place the allergens d2 and k82 and the Ligand-Labeled Anti- IgE Antibody in appropriate racks on the Mark 5.

(3) Place calibrators, controls and samples in the Mark 5 rack. Duplicate samples are pipetted in this order: calibration standards, negative control, positive control, and serum specimens. Duplicate positive and negative controls are run with each microplate.

(4) Two reservoirs for Reverse osmosis water should be full and connected to the Mark 5.

(5) The wastewater reservoir should be empty.

c. Mark 5 Specific IgE Parameters for patient samples: (machine turned on at all times)

Parameter	Setting
Volume (uL)	50
Number of Replicates	2
Number of Multishots	2
Excess Volume (%)	50
Aspirate Speed	7
Dispense speed	3
Dispense Height	256
Inter Transfer Wash Cycles	3

d. Max 002 formatting

(1) Turn on MAX 002 to ignite the 12 V lamp and start the computer.

(2) Fill Reservoir B with Reverse osmosis water.

(3) Fill Reservoir A with freshly prepared AlaSTAT Wash Solution.

(4) Empty waste bottle.

(5) Purge manifold with Reverse osmosis water to ensure that none of the wash needles are blocked. From the Main Menu at System Ready select [Washer] [Manual] Util][Purge] [Fluid B]. Rinse with 2500 uL. Check that all needles dispense water in a smooth and equal fashion.

(6) Purge Multi-Reagent Dispenser (MRD) Module with Reverse osmosis water to check that lines are clear [MRD] [Start] [test number 48]. Likewise purge with air at least twice.

(7) Fill MRD bottles with appropriate reagents as follows:

Line A connected to reagent Anti-Ligand

Line C connected to TMB Substrate solution

Line E connected to Enzyme-Labeled Anti-IgE Antibody

e. MAX 002 Spectrometric Setting:

A = anti-ligand

C = substrate

E = enzyme for Specific IgE

W = bottle A wash solution

Parameter	Setting
1st dispense	0
1st dispense fluid	-
1st purge	-
1st incubation	60 minutes
2nd dispense	50 microliters
2nd dispense fluid	A
2nd purge	200 microliters x 4
2nd incubation	60 minutes
1st wash cycle	2
1st wash	380 microliters
1st wash fluid	A
1st shake	0
1st aspirate ALL	NO
3rd dispense	200 microliters
3rd dispense fluid	E
3rd purge	200 microliters x 2
3rd incubation	60 minutes
2nd wash cycle	4
2nd wash	380 microliters
2nd wash fluid	A
2nd shake	0
2nd aspirate ALL	NO
4th dispense	200 microliters
4th dispense fluid	C
4th purge	200 microliters x 3
First plate shake	10 seconds
Test filter	650 nm
Read period	5 minutes
Calculation mode	linear regression
O.D. limit	2.500
Washer clean volume	2,000 microliters
Washer clean fluid	Reverse osmosis water
Washer purge volume	1,000 microliters of wash fluid

f. Operation

(1) Select AlaSTAT /Pipet from the WinMAX Worklist Editor of the WinMAX program.

(2) Select Specific IgE from the Assay list box.

(3) Click the Operator text box and enter the name of the operator.

(4) Click OK. WinMAX displays the layout required to process the assay.

(5) Review the required assay components to make sure that there is sufficient quantity on the Mark 5 deck to complete the run.

(6) Click GO to start pipetting the assay. During pipetting, the Mark 5 Runtime Display depicts the progress of the instrument as it pipettes the various components, coloring the run-time display representations of the microwells as the Mark 5 pipettes to them. The Mark 5 allows reagents to be replenished as needed before pipetting is completed.

(7) At the completion of the pipetting cycle, the Mark 5 screen 5displays Mark 5 Pipetting Completed on the screen.

(8) All reagents used on the Mark 5 and the patient samples are removed from the Mark 5 and placed at 4º C.

(9) Perform an Extended Prime with Reverse osmosis water, then with Delta Clean, and then three more times with Reverse osmosis water.

(10) Microplates are removed from the Mark 5 to continue their processing on the MAX 002.

(11) MAX 002 display will read Test Name: AlaSTAT-M for the start of a Specific IgE assay.

(12) Press [^] key under Enter.

(13) Enter operator ID if desired. (Use arrows to scroll through alphabet.)

(14) Screen will display Remove clips from under pinch valves, Check reagent level and seals, and Checking pressure. Press Enter after each display after each parameter is met.

(15) The MAX 002 will prompt the operator to place the microplates on the MAX 002. This must be done with the plates in the proper orientation and order.

(16) The MAX 002 will then process all plates without user intervention.

(17) When assay has completed Collect MAX will appear on the computer screen. Click OK.

(18) Under AlaSTAT select Data Reduction. The computer screen will appear showing the mOD/MIN
generated on the first plate.

(19) To print the Assay Report, click on the Printer icon and select Assay Report. OK

(20) Curve and results will print out.

(21) Remove all reagents from the MAX and store at 4º C.

(22) Flush lines with Reverse osmosis water to check for blockages. Use small wire to clear any blockage. Repeat Reverse osmosis water flush until all lines run freely.

(23) Flush lines with air at least twice.

(24) Turn off the MAX 002 and protect with dust cover.

g. Recording of Data

(1) Quality control data. Use the “HANES LABORATORY IgE STANDARD AND QUALITY CONTROL” format in an EXCEL program to record this data. The reporting sheet has blanks for the following:

Run Number A
Run Date A
Negative Pool Value A
Negative Mean A

Negative Std. Dev. A
Positive Pool Value A
Positive Std. Dev. A
Positive Mean A

(2) Analytical results. Use the "HANES 99+ ANALYTICAL WORKSHEET" to record the specimen results using an EXCEL program. The recording sheet has blanks for the following:

Sample ID
Slot Number
Sample Collection Date
MEC Comment Code
Run Number A
Run Date A
Tech ID A
Analyte Result A
Analyte Class A
Comment A
Repeat Run Number A
Repeat Run Date A
Repeat Analyte Result A
Repeat Analyte Class A

(3) Give both types of forms to the supervisor along with the computer printout from the calculation program. After the supervisor checks the data, the analyst's copies and the printouts should be returned for filing in a notebook. The supervisor will keep the original copies of the reporting information.

(4)The data is sent to NHANES in the same format as shown in (1), but delineated by commas.

h. Replacement and Periodic Maintenance of Key Components

(1) 12 Volt lamp: A spare lamp should be available. Order another spare if used for replacement.

(2) Printer tape: A supply of printer tape should be on hand.

i. Calculations

(1) A linear regression program is used by the WinMAX program to calculate the calibration curve and the specimen concentrations.

(2) Repeat a specimen analysis when duplicate values do not check within 10%.

j. Special Method Notes

Once the samples have been pipetted into the ligand-coated microplate, continue through the analysis without interruption to minimize errors due to interference in the binding of components.

8. REPORTABLE RANGE OF RESULTS

(1) The lower limits of reporting, using the WinMAX System, is less than 0.35 kilounits of IgE per liter.

(2) The Class number is a semiquantitative index to the amount of endogenous IgE specific for the selected allergen, in this case, Specific IgE. Qualitative values and interpretation of Class results are as follows:

Class	kU/L	Interpretation
0	<0.35	Negative for Specific IgE

I	0.35 – 0.69	Positive for Specific IgE
II	0.70 – 3.49	Strongly positive for Specific IgE
III	3.50 – 17.49	Strongly positive for Specific IgE
IV	17.5 - 52.49	Strongly positive for Specific IgE
V	52.5 – 99.9	Strongly positive for Specific IgE
VI	≥100	Strongly positive for Specific IgE

9. QUALITY CONTROL (QC) PROCEDURES

Specific IgE is measured by a batch method (i.e. all specimens, standards, and QC specimens undergo the same processes, such as automated pipetting, incubating, washing, addition of reagents). On an average day, 86 specimens are analyzed in duplicate, with 2 levels of QC specimens on each microplate. Two controls are included on each microplate, a negative control and a positive control.

The system is declared “out-of-control” if any of the following events occur:

- The negative control result is greater than 0.34.
- The positive control result is not between 1.0 and 2.0.
- The calibration curve is not linear.

10. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If one or more quality control samples fall outside the acceptable range of duplicate values, take the following steps:

a. Open a new control vial of the out-of-control reagent, and reanalyze the samples using the fresh control.
b. Open a box of fresh calibration standards, and repeat the entire curve using the fresh standards.

If the steps outlined above do not result in the correction of the “out of control” values for QC and calibration materials, the supervisor should be consulted for other corrective actions. No analytical results should be reported for runs not in control.

11. LIMITATION OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. A definitive clinical diagnosis should not be made solely on the basis of an in vitro allergen-specific IgE result. Diagnosis should be made by the physician only after all clinical and laboratory findings have been evaluated.[2,4]
b. In vitro allergen-specific IgE results should not be used as a definitive guide to selecting an initial dose for immunotherapy; a skin test with the proposed initial dilution of the allergenic extract should be performed first to demonstrate the patient’s ability to tolerate the dose.
c. In food allergy, circulating IgE antibodies may remain undetectable if directed towards allergens which are revealed or altered during processing or digestion and which therefore do not exist in the original food for which the patient is tested.[1,3]
d. Identical results for different allergens may not be associated with clinically equivalent manifestations, due to differences in IgE-binding capacity.[7]
e. This test is not intended for monitoring purposes; however, when evaluating a series of samples from a given individual for changes over time, all samples should be processed in a single assay.[6]
f. The user should be aware of the possibility of clinical crossreactivity within an allergen family.[8,9]
g. The following special considerations apply to latex allergy testing:

- The possibility of clinical crossreactivity exists between latex and certain foods including avocado, banana, chestnut, and kiwi.[15]

- Since the latex assay measures allergen-specific IgE, type IV delayed reaction or irritation from latex will not be detected.

h. AlaSTAT Microplate class 0 results for insect venoms indicate absent or undetectable levels of circulating venom-specific IgE antibodies. Such results do not preclude existence of current or future clinical hypersensitivity to insect sting.

12. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are stored frozen at -70º C until they are tested.[16] Specimens equilibrate to room temperature on the day of testing.

13. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no acceptable alternative methods for performing this test. In case of system failure, store all serum specimens at -70º C until the system is functioning.

14. PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

All records, including related QA/QC data, are stored in EXCEL files. Only numerical identifiers should be used.

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19. Summary Statistics and QC Graphs

Summary Statistics for Latex by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
008	3	11/19/1999	11/23/1999	1.692	0.129	7.630
009	42	11/29/1999	8/15/2000	1.635	0.135	8.280
010	12	8/31/2000	12/4/2000	2.488	0.180	7.223