For Individual Laboratory to Complete:

Laboratory Name		
Adopted		
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anti-dsDNA
Enzyme Immunoassay

Method: Diamedix Corp., *immuno*simplicity®

Manual or in conjunction with one of the Diamedix Automated EIA Systems such as the MAGO Plus, the DSX, or the DS2. For *In Vitro* Diagnostic Use.

Clinical Significance

A hallmark of the systemic rheumatic diseases has been the presence of circulating serum antibodies to various nuclear antigens(1). Autoantibodies to nuclear antigens (ANA) include antibodies to all antigens present in the nucleus such as DNA, histones, non-histones and nucleoli. Autoantibodies to dsDNA are important and characteristic markers in patients with SLE and their appearance serves as an important tool in the diagnosis, prognosis, and monitoring of SLE patients. Antibodies to dsDNA occur in approximately 60-70% of SLE patients and there is considerable evidence to implicate immune complexes containing anti-dsDNA and DNA in the pathogenesis of SLE(1,2).

Low levels of anti-dsDNA antibodies may occur in other rheumatic diseases (3) and may occur at a very low frequency (2-3%) in individuals without any symptoms of rheumatic disease (4). It has also been reported that the appearance of anti-dsDNA in rheumatic patients can occur prior to the development of the complete clinical pattern(5).

A number of techniques have been developed to detect anti-dsDNA antibodies. In the past, the most utilized tests have been the *Crithidia luciliae* immunofluorescent assay, and the Farr radioimmunoassay. However, the sensitivity, specificity, precision and ease of performance of these assays can vary considerably. The enzyme-linked immunosorbent assay (EIA) offers advantages over these methods in terms of sensitivity, reproducibility, objectivity and potential for automation. The usefulness of EIAs in anti-DNA determinations is widely documented and accepted (6,7,8).

The Diamedix *immuno*simplicity® (Is)-anti-dsDNA Test Kit is an EIA procedure intended for the quantitation of IgG antibodies to dsDNA. The results are reported in IU/ml, traceable to the First International Standard for antibodies to double stranded DNA (Wo/80) (9). The test can be performed either manually or in conjunction one of the Diamedix Automated EIA Systems.

Principle of the Procedure

Diluted samples are incubated with plasmid DNA (grown in E. coli and purified by alkaline hydrolysis and chromatography) bound to the solid surface of a microwell. If IgG antibodies against DNA are present in the samples, they will bind to the antigen forming antigen-antibody complexes. Residual sample is eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG) is added and will bind to these complexes. Unbound conjugate is removed by aspiration and washing. Substrate is then added and incubated. In the presence of bound enzyme the substrate is converted to a colored end product. The absorbance of this end product can be read spectrophotometrically at 450 nm (reference 600-630 nm) and is directly proportional to the concentration of IgG antibodies to dsDNA present in the sample.

Specimen Collection

Whole blood should be collected by accepted medical techniques. Separated serum should remain at 22°C for no longer than 8 hours. If assays are not completed within 8 hours, serum should be refrigerated (2 to 8°C). If assays are not completed within 48 hours,samples should be frozen at -20°C. Avoid multiple freeze-thaw cycles. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Grossly contaminated, hemolyzed, lipemic, or icteric specimens should not be used. The NCCLS provides recommendations for collecting and storing blood specimens (10).

CAUTION: Serum samples must not be heat-inactivated prior to use.

Reagents

Antigen Wells Twelve, 8-well microwell breakapart strips,color-coded orange, coated with purified plasmid DNA.

0 IU/ml Standard One vial with yellow cap containing 0.25 ml human serum or defibrinated plasma, non-reactive for dsDNA IgG antibodies,and 0.1% sodium azide. Assigned IU/ml value printed on label.

12.5 IU/ml Standard One vial with green cap containing 0.25 ml human serum or defibrinated plasma, with the assigned level of dsNA IgG antibodies,and 0.1% sodium azide. Assigned IU/ml value printed on label.

25 IU/ml Standard One vial with brown cap containing 0.25 ml human serum or defibrinated plasma, with the assigned level of dsDNA IgG antibodies,and 0.1% sodium azide. Assigned IU/ml value printed on label.

50 IU/ml Standard One vial with purple cap containing 0.25 ml human serum or defibrinated plasma, with the assigned level of

dsDNA IgG antibodies,and 0.1% sodium azide. Assigned IU/ml value printed on label.

100 IU/ml Standard One vial with white cap containing 0.25 ml human serum or defibrinated plasma, with the assigned level of dsDNA IgG antibodies,and 0.1% sodium azide. Assigned IU/ml value printed on label.

200 IU/ml Standard One vial with red cap containing 0.25 ml human serum or defibrinated plasma, with the assigned level of dsDNA IgG antibodies,and 0.1% sodium azide. Assigned IU/ml value printed on label.

Positive Control One vial with blue cap containing 0.25 ml human serum or defibrinated plasma reactive for dsDNA IgG antibodies, 0.1% sodium azide. Assigned IU/ml range printed on label. The Positive Control is used to control the lower to mid-range of the assay.

Negative Control One vial with black cap containing 0.25 ml of human serum or defibrinated plasma non-reactive for dsDNA antibodies, and 0.1% sodium azide. Assigned IU/ml range printed on the label. The Negative Control is used to control the negative range of the assay.

Sample E Diluent One bottle with blue cap containing 60 ml Phosphate buffer with Tween 20 and protein stabilizers. Contains Proclin™ 300, 15 ppm active ingredient. Color coded blue.

Wash U Concentrate (20X) Two bottles with clear caps containing 50 ml of Phosphate buffer with detergent and Proclin™ 300, 15 ppm active ingredient . Each bottle is sufficient to make 1 liter of wash solution.

Conjugate One bottle with red cap containing 25 ml goat anti-human immunoglobulin G labeled with horseradish peroxidase. Also includes protein stabilizers and Proclin™ 300, 30 ppm active ingredient. Color-coded pink.

Substrate HRP One amber bottle with brown cap containing 25 ml buffered TMB solution (3,3',5,5' tetramethylbenzidine).

Stop O Solution One bottle with white cap containing 30 ml of 1 N Hydrochloric acid. **CAUTION:** Acids are corrosive. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water.

These reagents should be stored at 2 to 8° C.

Other Materials Required

Manual Users:

1. Wash bottle or automated microplate washer
2. Pipettors capable of dispensing appropriate volumes
3. Timer
4. One liter graduated cylinder
5. One liter wash solution reservoir
6. Deionized or distilled water
7. Absorbent toweling
8. Tubes or microwell plate for serum dilution
9. Reader capable of reading absorbance at 450nm, reference at 600 or 630 nm.

Diamedix Automated EIA System Users:

1. One liter graduated container
2. Deionized or distilled water
3. Dilution containers as appropriate to system.
4. Sample & Reagent tips required by system.
5. Reagent containers required by system.

Warnings:

1. Handle samples, Standards, controls and the materials that contact them as potential biohazards. Each donor unit in the standards and controls has been found negative for Hepatitis B surface antigen and HIV-I antibodies by FDA-approved third generation tests. However, because no method can offer complete assurance that HIV-1, Hepatitis B virus, or other infectious agents are absent, these materials should be handled at the Biosafety Level 2 as recommended for any potentially infectious serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories", 1993.
2. Never pipette by mouth.
3. Avoid contact with open skin and mucous membranes.
4. Certain of the test reagents contain Proclin™ 300 as a preservative. When disposing of reagents containing Proclin™ 300 , flush drains with copious amounts of water to dilute the active components below active levels.
5. Serum components contain sodium azide as a preservative. Azides are reported to react with lead and copper in plumbing to form compounds that may become explosive. When disposing of solutions containing sodium azide, flush with copious amounts of water to minimize the build up of metal azide compounds.

Calibration

This test can use a 6-point calibration system (with single-point calibration optional)based on reference standards. These standards have been prepared from serum that is strongly positive for the antibody under investigation. The Standards have been assigned unitages in International

Units(IU) per ml, traceable to the First International Standard for antibodies to dsDNA (Wo/80),WHO Reference preparation (9). The Standards (or 200 IU/ml Standard for the single-point option) are(is) included in every test run and are(is) diluted and run in the same way as the test samples.

The upper end of the reportable range was determined to be 200 IU/ml and a Standard is prepared at that level. The 0 IU/ml Standard is prepared from material devoid of the antibody in question. The test can be performed using all 6 Standards (no blank required) and reading the results from a point-to-point standard curve produced.

This test can also use single point calibration. This is possible because the dose response curve is sufficiently linear and passes near to, or through the origin. The linearity of the dose response has been validated by the manufacturer during quality control testing. For a single-point curve, only the 200 IU/ml Standard is used. A blank (Sample Diluent only) is required in the first well.

NOTE: Positive and Negative Controls must be run for either assay option.

Patient samples which contain very high levels of antibody may produce absorbance values greater than that of the highest Standard. Such patient sample results should be reported as "Greater than 200 IU/ml". If numerical results are required for such samples, dilute the sample using Sample Diluent and re-assay. Several dilutions (for example 1/10, 1/50 and 1/100) of the sample may be re-assayed simultaneously. Select the dilution that has an absorbance reading about 50% of the absorbance reading of the Calibrator; calculate the IU/ml for this dilution and multiply by the dilution factor to obtain estimated values.

Quality Control

a) The Positive and Negative Controls must be included in each test run.
b) The absorbance of the Blank or the 0 IU/ml Standard must be <0.2.
c) The Positive and Negative Controls must be within their assigned ranges.
d) The absorbance of the Negative Control must be lower than that of the 25 IU/ml Standard.
e) The absorbance of the Positive Control must be higher than that of the 25 IU/ml Standard.
f) The absorbance of the 200 IU/ml Standard must $\geq$ 3 times the absorbance of the 25 IU/ml Standard.

If any of these criteria is not met, the run is invalid and must be repeated.

NOTE: Additional controls may be tested according to guidelines or requirements of local, state or federal regulations or accrediting organizations. For guidance on appropriate quality control practices,

please refer to NCCLS document C24-A, Internal Quality Control Testing:Principles and Definitions.

Procedure

Allow all test components and patient samples to warm to room temperature before use. Invert reagent bottles gently several times before use. Return promptly to the refrigerator after use.

Prepare Wash Solution by adding 50 ml of Wash Concentrate(20X) to one liter of deionized or distilled H_2O.

1. Prepare 1:101 dilutions of the Standard(s), controls and patient samples in Sample Diluent. (e.g., by addition of 5 µl sample to 500 µl Sample Diluent).

2. Mix dilutions gently by withdrawing and expelling in a pipette tip 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 µl of diluted Standard(s), controls and patient samples to the antigen wells. Avoid formation of bubbles when transferring diluted samples.

3. ***NOTE:*** *For the single-point calibration option, include one well which contains 100 µl of Sample Diluent only as the reagent blank. This will ultimately be used to "zero" the photometer before reading the test results.*

4. Allow the wells to incubate uncovered at room temperature(18-30°C) for 30 ± 5 minutes.

5. Aspirate or discard the contents of the wells. Remove any excess moisture in the wells by tapping on paper toweling if necessary. Wash the wells by rinsing 3 times with at least 300 µl per well of Wash Solution. Remove excess moisture from the wells after washing. When using an automated washer, follow the manufacturer's instructions.

6. Place 100 µl of Conjugate into each well, avoiding bubble formation.

7. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 ± 5 minutes.

8. Wash the wells as described in Step 5 above.

9. Place 100 µl of Substrate into each well, avoiding bubble formation.

10. Allow the wells to incubate uncovered at room temperature(18-30°C) for 30 ± 5 minutes.

11. Place 100 µl of Stop Solution into each well, avoiding bubble formation.

12. Read the absorbance of each well at 450 nm using a reference wavelength of 600-630 nm. For single-point option, zero against the reagent blank. Read the plate within 60 minutes of adding Stop Solution.

Refer to the BP-96 Plate Reader (or equivalent) Operation Manual for complete instructions on set-up and operating procedures.

Diamedix Automated EIA System Users:
If using one of Diamedix's Automated EIA System, please refer to the corresponding Operating Manual for the test setup, procedure, and accessories/consumables needed.

Calculation of Results

Since the dose response curve for the Is anti-dsDNA Test Kit is sufficiently linear, results can be calculated using either a Single Point Calibrator only or, if the user wishes, a 6-Point Caliobration system. The results obtained by the Single Point or 6-Point Calibration Methods have been shown to have a correlation coefficient >0.99 (data on file).

1. Single Point Calibration

Determine the IU/ml (International Units/ml) for each patient specimen or control using the following formula:

$$\frac{\text{Absorbance Of Sample}}{\text{Absorbance of 200 IU/ml Standard}} \times 200 = \text{IU/ml of sample}$$

2. 6-Point Calibration

Results are obtained from the best linear curve fit using all six Standards. For the Stat-Fax® 2100 readers (or equivalent)the Regression Mode should be selected and Standard values entered accordingly. The Diamedix Automated EIA Systems will calculate results automatically for either option.

3. Interpretation

The following is a guide to interpretation of results. **Each laboratory can establish its own "normal" ranges based on populations encountered.**

< 25 IU/ml	Negative for antibodies to dsDNA.
25.0-34.9 IU/ml	Equivocal for antibodies to dsDNA. Sample should be retested. If retest results are equivocal, the sample should be reported as equivocal, tested by another method, or a new sample should be tested.**
35.0-99.9 IU/ml	Weakly positive for antibodies to dsDNA.
100-200 IU/ml	Moderately positive for dsDNA antibodies.
≥ 200 IU/ml	Strongly positive for dsDNA antibodies.

**Equivocal samples that give positive results on retest should be reported as positive. Equivocal samples that give negative results on retest should be reported as negative.

Procedure Notes

1. Sodium azide inhibits horseradish peroxidase activity. Care must be taken to ensure that azide is not carried over from other reagents into conjugate and substrate steps.

2. Avoid contamination of the TMB substrate solution with conjugate or other oxidants which will cause the solution to change color prematurely.

3. The substrate contains 3,3’5,5’ tetramethylbenzidine (TMB) which has shown possible mutagenic effects in laboratory experiments.

4. Do not interchange reagents from different reagent lots except for Sample **E** Diluent, Wash **U** Concentrate, Substrate **HRP** and Stop **O** Solution may be interchanged between lots.

5. Do not use reagents beyond their expiration date.

6. Store unused reagents at 2 to 8°C results.

7. Incubations above or below the recommended temperatures or times may give erroneous results.

8. The EIA method is a very sensitive technique. Maintain consistent pipetting technique, incubation times, and temperature conditions throughout the test procedure. Cross contamination between reagents can invalidate the test.

9. Antigen coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.

10. *(Manual Procedure Only)* The washing procedure is very important and requires special attention. (Please refer to the Procedure section)

    NOTE: *Improperly washed* wells *may* give *erroneous results.*

11. The reported concentration of anti-dsDNA in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

Limitations

1. The results obtained with the Is-anti-dsDNA IgG test kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves but used in conjunction with clinical findings and other serological tests.

2. The test should be performed on serum. The use of whole blood or plasma has not been established.

3. Assay performance characteristics have not been established for visual result determination.

4. The analysis of a single serum sample should not be used as the sole criterion for diagnosis of an autoimmune disease.

5. Screening of the general population should not be performed. The positive predictive value depends on the likelihood of autoimmune disease being present. Testing should be performed only when clinical symptoms are present or disease is suspected.

6. In approximately 30-40% of lupus patients, anti-dsDNA antibodies are undetectable.

7. The presence of low levels of antibodies to dsDNA may occur in other autoimmune diseases, may antedate the onset of clinical symptoms of SLE, may represent the period following exacerbation of symptoms or may reflect remission or control.

8. A small and variable percentage of people have antibodies to dsDNA in the absence of disease.

9. The performance characteristics of the Diamedix Is-anti-dsDNA IgG Test Kit with automated equipment other than one of Diamedix’s Automated EIA Systems have not been established.

References

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