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For Individual Laboratory to Complete:

Rheumatoid Factor Enzyme Immunoassay

Laboratory Name		
Adopted		
Reviewed		
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Supercedes		

Method: Diamedix Corp., Immunosimplicity®

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

Rheumatoid arthritis (RA) is a chronic relapsing inflammatory arthritis of unknown etiology usually affecting multiple joints with a varying degree of systemic involvement. The disease has been estimated to occur in 1 to 2% of the general population. Females are affected more than males by a three to one margin and prevalence increases with age, peaking at 35-45 years of age (1,2).

A characteristic of RA is the presence in the blood and synovial fluid of a reactive group of proteins collectively known as Rheumatoid Factors (RF). Rheumatoid Factors are immunoglobulins of any isotype with antibody activity directed against antigenic sites in the Fc region of human or animal immunoglobulin G antigen, IgM-RF is the main isotype identified by clinically available diagnostic tests for RF detection (3). RF has been reported to occur in approximately 70-80% of patients with confirmed RA (4,5). The concentration of RF tends to be highest when the disease peaks and tends to decrease during prolonged remission. This high RF frequency in RA cases makes their detection useful as a diagnostic tool, however, these factors are not unique to RA. RF is found in approximately 4% of the general population. RF is present in 75% of adult patients, with the highest incidence of RF occurring in patients over 65 years of age and nearly all patients with Felty and Sjogren's Syndrome. Increased titers may accompany a variety of acute immune responses, particularly viral infections and a number of other diseases (infectious mononucleosis, tuberculosis, leprosy, various parasitic diseases, liver disease, sarcoidosis and systemic lupus erythematosus) (6,7).

Conventional methods for the measurement of RF-IgM have depended upon the agglutination of particles (latex, charcoal, bentonite or erythrocytes) coated with human or animal IgG. The latex agglutination test is sensitive but it can result in a fairly high number of false positives. Quantitative serological tests such as EIA provide an objective measurement on a single sample dilution (8).

The Diamedix Immunosimplicity® Is-Rheumatoid Factor Test Kit is an EIA procedure intended for the quantitation of IgM antibodies to IgG antigen. The results are reported in IU/ml, traceable to the WHO International Reference preparation (9). The test can be performed either manually or in conjunction with one of the Diamedix Automated EIA Systems.

Principle of the Procedure

Diluted samples are incubated with purified RF antigen (human IgG) bound to the solid surface of a microtiter well. Any RF-IgM antibody present binds to the immobilized human IgG to form antigen-antibody complexes. Unbound antibody is washed from the wells and horseradish peroxidase-conjugated anti-human IgM is added. The enzyme conjugate binds to the antigen-antibody complex. Excess conjugate is washed away and a specific substrate added. Bound enzyme conjugate begins a hydrolytic reaction causing color development. After a specific time, the reaction is stopped. The intensity of the generated color is proportional to the amount of RF specific IgM antibody bound to the wells. The results are read on a spectrophotometer. The net absorbance is calculated by subtracting the absorbance values for the blank from the absorbance value for the sample. A calibrator that is assayed with each run is then used to calculate the RF-IgM activity in the IU/ml from the net absorbance value.

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 at 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 pink, coated with purified human IgG.
Calibrator	One vial with blue cap containing 0.25 ml of pre-diluted human serum, highly reactive for RF-IgM antibodies, 0.1% sodium azide. Assigned IU/ml value printed on label.
Positive Control	One vial white cap containing 0.25 ml of pre-diluted human serum, moderately reactive for RF-IgM antibodies, 0.1% sodium azide. Assigned IU/ml range printed on label.
Negative Control	One vial with black cap containing 0.25 ml of pre-diluted human serum, non-reactive for RF-IgM antibodies, 0.1% sodium azide.
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 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 M labeled with horseradish peroxidase. Also

includes protein stabilizers and preservatives. Color-coded pink.

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

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. See precautions section.

Store these reagents 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 and Reagent tips required by system
5. Reagent containers required by system

Warnings:

1. Handle samples, Calibrator, 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, HCV and HIV-1 & 2 antibodies by FDA-approved third generation tests. However, because no method can offer complete assurance that HIV-1 & -2, Hepatitis B virus or Hepatitis C 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. The concentrations of RF IgM-class antibodies in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.
3. Never pipette by mouth.
4. Avoid contact with open skin and mucous membranes.
5. Certain of the test 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.

6. Certain of the test reagents contain Proclin™300 as a preservative. When disposing of these reagents, flush drains with copious amounts of water to dilute the active components below active levels.
7. Sodium azide inhibits horseradish peroxidase activity. Care must be taken to ensure that azide is not carried over from other reagents into the immunocomplex and substrate steps.
8. Avoid contamination of the TMB substrate solution with conjugate or other oxidants which will cause the solution to change color prematurely.
9. The substrate contains 3,3',5,5' Tetramethylbenzidine (TMB) which has shown possible mutagenic effects in laboratory experiments.

Calibration

This test uses an in-house reference standard (or Calibrator). This Calibrator has been prepared from a pool of sera strongly positive for the antibody under investigation. The Calibrator functions as an internal reference preparation and is assigned a unitage in International units (IU) per ml traceable to the WHO International Reference preparation. The Calibrator is included in every test run and is diluted and run in the same way as a test sample.

These tests have been optimized to permit the use of single point calibration. This is possible because the dose response curves are sufficiently linear and pass near to, or through the origin. The linearity of the dose response has been validated by the manufacturer during quality control testing.

Patient samples which contain very high levels of antibody may produce absorbance values greater than the Calibrator absorbance. Patient sample results greater than the Calibrator value should be reported as "Greater than 100 IU/ml". If numerical results are required for such samples, dilute the sample using Sample Diluent and re-assay. Several dilutions (for example 1/5, 1/10 and 1/20) of the pre-diluted 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

The Positive and Negative Controls must be included in each test run.

The absorbance of the Blank must be <0.2.

The Positive Control must be within its assigned range.

The Negative Control must be <16 IU/ml.

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

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 H₂O.

Manual Users:

1. Prepare 1:101 dilutions of the Calibrator, 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

Calibrator, controls and diluted patient samples to the coated wells. Avoid formation of bubbles when transferring diluted samples.

NOTE: 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.

3. Allow the wells to incubate at room temperature (18-30°C) for 30 ± 5 minutes.
4. 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.
5. Place 100 µl of Conjugate into each well, avoiding bubble formation.
6. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 ± 5 minutes.
7. Wash the wells as described in Step 4 above.
8. Place 100 µl of Substrate into each well, avoiding bubble formation.
9. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 ± 5 minutes.
10. Place 100 µl of Stop Solution into each well, avoiding bubble formation.
11. Read the absorbance of each well at 450 nm and zero against the reagent blank. A suitable reference wavelength (e.g., 600-630 nm) reading should be used. Read the plate within 30 minutes of adding Stop Solution.

Diamedix Automated EIA System Users:

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

Calculation of Results

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

$$\frac{\text{IU/ml of Calibrator}}{\text{Absorbance of Calibrator}} \times \text{Absorbance of Test Sample} = \text{IU/ml of sample}$$

When using one of the Diamedix Automated EIA Systems, results are automatically calculated.

Reference Ranges

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

Less than 16 IU/ml Index < 0.80	Negative for RF.
Greater than/equal to 20 IU/ml Index \geq 1	Positive for RF.
16 to 19.9 IU/ml Index 0.80-0.99	Equivocal* for RF.

* If equivocal results are obtained, samples may be re-tested, another sample should be obtained or the sample may be tested by an alternate method.

Procedure Notes

1. The following components are interchangeable: Sample **E** Diluent, Wash **U** Concentrate, Substrate **HRP**, and Stop **O** Solution.
2. Do not use reagents beyond their expiration date.
3. Store unused reagents at 2 to 8°C results.
4. Incubations above or below the recommended temperatures or times may give erroneous results.
5. 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.
6. RF-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.

Limitations

1. A negative result does not exclude rheumatoid arthritis. Approximately 25% of patients with a diagnosed case of rheumatoid arthritis may present with a negative result for RF.
2. Certain non-rheumatoid conditions, connective tissue disorders and a variety of other diseases such as hepatitis may elicit a positive RF test.
3. RF exists in three major immunoglobulin classes: IgA, IgG and IgM. Most test systems for RF are designed to detect IgM-RF because the molecules are large and react more easily with human IgG coated on the solid phase of the test system. Consequently, these tests will only detect RF of the IgM class.
4. The test should be performed on serum. The use of whole blood or plasma has not been established.

5. The performance characteristics of the Diamedix Is-Rheumatoid Factor Test Kit with automated equipment other than the Diamedix Automated EIA Systems have not been established.

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