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For Individual Laboratory to Complete:

**Rubella IgG
Enzyme Immunoassay**

Laboratory Name		
Adopted		
Reviewed		
Reviewed		
Revised		
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

Rubella (german or '3-day' measles) is a mild, contagious rash primarily of children and young adults. Acute rubella virus infection in a child or adult is usually a self-limited, benign disease, characterized by a low-grade fever, mild upper respiratory symptoms, an erythematous maculopapular rash and suboccipital lymphadenopathy. However, rubella can be a very serious disease early in pregnancy, leading to miscarriages, stillbirths or birth defects (congenital rubella syndrome, or CRS). Common manifestations of congenital rubella include deafness, ocular problems including cataracts and glaucoma, congenital heart disease and mental retardation (1, 2).

The severity and risk of the effects of rubella virus on the fetus depend on the time during pregnancy when the rubella infection occurs. Up to 85% of infants infected in the first trimester will be found to be affected after birth and even an inapparent rubella infection in the mother can result in birth defects. After an attack of rubella or vaccination against rubella most mothers are protected against the disease for life. However, reinfection with rubella can occur (3,4). Reinfection occurs more frequently in vaccinated than in naturally immune individuals (5). The overwhelming majority of these reinfections occur without symptoms. Rubella reinfection during pregnancy, however, rarely results in transmission of the virus to the unborn child (2,3).

Since rubella vaccines were first licensed for use in 1969 the number of reportable cases has dropped dramatically. However, in recent years a moderate resurgence of rubella has occurred. Although rash is the most conspicuous feature of the disease, it is of such a variable character that it may be confused with that produced by other infectious diseases and even by drugs. Thus, diagnosis of rubella on clinical grounds may be somewhat inaccurate and there is a need for continued surveillance to identify susceptible individuals and reduce the risk of CRS (2,3).

Serologic techniques for the detection of antibodies to rubella virus provide the approach of choice for the laboratory diagnosis of acute and congenital rubella infections and for the determination of rubella immune status. The presence of IgM antibody or a significant rise in IgG for acute and convalescent specimens is evidence of acute rubella infection. The acute phase specimen should be drawn as soon after rash onset as possible; the convalescent-phase serum should be drawn 10 or more days after the acute-phase specimen (3,6).

Historically, hemagglutination inhibition (HI or HAI) has been the most frequently used method of screening for the presence of rubella antibodies. The first enzyme immunoassay (EIA) for rubella was reported in 1975 (7) and since then this method has gained widespread acceptance.

The Diamedix Immunosimplicity[®] Is-Rubella IgG Test Kit is an EIA procedure intended for the qualitative and quantitative detection of antibodies to rubella antigen. The test can be performed either manually or in conjunction with Diamedix Automated EIA Systems. The results are reported in IU/ml, which are traceable to the WHO 1st International Standard for Anti-Rubella Immunoglobulin, Human, 1996 (8).

Principle of the Procedure

Diluted samples are incubated rubella antigen bound to the solid surface of a microtiter well. If IgG antibodies against rubella 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 an 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 rubella antigen 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, or the separated sample is to be stored beyond 48 hours, samples are to be frozen at -20°C. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen sera to room temperature slowly and mix gently, avoiding foam formation. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Grossly contaminated, hemolyzed, lipemic, or icteric specimens should not be used.

For the diagnosis of acute rubella infection, the acute-phase serum sample should be drawn as soon as possible after rash onset, preferably within

the first 7 days. The convalescent-phase sample should be drawn 10-14 days after the acute-phase specimen.

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

Reagents

Antigen Wells	Twelve, 8-well microwell breakapart strips, color-coded purple, coated with grade IV sucrose purified rubella antigen (strain HPV 77 produced in Vero cells).
0 IU/ml Standard	One vial with yellow cap containing 1.8 ml of pre-diluted human serum, non-reactive for Rubella IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned IU/ml value printed on label.
10 IU/ml Standard	One vial with green cap containing 1.8 ml of pre-diluted human serum, weakly reactive for Rubella IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned IU/ml value printed on label.
50 IU/ml Standard	One vial with red cap containing 1.8 ml of pre-diluted human serum, moderately reactive for Rubella IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90ppm active ingredient. Assigned IU/ml value printed on label.
High Positive Control	One vial with white cap containing 1.8 ml of pre-diluted human serum, highly reactive for Rubella IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned IU/ml range printed on label.
Low Positive Control	One vial with blue cap containing 1.8 ml of pre-diluted human serum, weakly reactive for Rubella IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Assigned IU/ml range printed on label.
Negative Control	One vial with black cap containing 1.8 ml of pre-diluted human serum, non-reactive for Rubella IgG antibodies, 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient.

Note: Standards and Controls are prepared from different serum lots.

Sample A Diluent	One bottle with blue cap containing 60 ml Phosphate buffer with protein stabilizers. Contains 0.2% sodium azide and Proclin™ 300, 90 ppm active ingredient. Color-coded blue.
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- Wash S Concentrate** Two bottles with clear caps containing 50 ml of Phosphate buffered saline with Proclin™ 300, 15 ppm active ingredient. Color-coded light blue/green. 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 preservatives. Color-coded pink.
- Substrate HRP** One amber bottle with brown cap containing 25 ml buffered TMB solution (3,3',5,5' tetramethylbenzidine).
- Stop M Solution** One bottle with white cap containing 30 ml of 1 N Phosphoric and 1N Hydrochloric acids. **CAUTION:** Acids are corrosive. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water. See Precautions section.

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 600-630 nm.
10. Incubator capable of maintaining temperature of $37 \pm 3^{\circ}\text{C}$

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, 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 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. Sodium azide inhibits conjugate activity. Clean pipet tips MUST be used for conjugate addition so that azide is not carried over from other reagents.
7. Avoid contamination of the TMB substrate solution with conjugate or other oxidants which will cause the solution to change color prematurely.
8. The substrate contains 3,3' 5,5' Tetramethylbenzidine (TMB) which has been shown to cause possible mutagenic effects in laboratory experiments.

Calibration

This test uses a 3-point calibration system (with qualitative reporting 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 WHO 1st International Standard for anti-Rubella Immunoglobulin, Human, 1996 (8).

The positive cut-off has been assigned a value of 10 IU/ml and a Standard prepared at that level. The upper end of the reportable range has been assigned a value of 50 IU/ml and a Standard prepared at this level. The 0 IU/ml Standard is prepared from material devoid of the antibody in question. The test can be performed using all three Standards and reading the results from the point to point standard curve produced. If the user desires a qualitative result, i.e. negative or positive, then only the 10 IU/ml Standard needs to be tested and patient absorbance values obtained compared to the 10 IU/ml Standard absorbance.

Patient samples which contain high levels of antibody may exceed the absorbance of the highest Standard. Such patient sample results should be reported as "Greater than 50 IU/ml". If numerical results are required for such samples, pre-dilute the sample using Sample Diluent and re-assay.

The resulting IU/ml value should then be multiplied by the dilution factor to obtain estimated values.

Quality Control

For Quantitative Assays

- a) The High Positive, Low Positive and Negative Controls must be included in each test run.
- b) The absorbance of the 0 IU/ml Standard must be < 0.2 .
- c) The absorbance of the 10 IU/ml Standard must be higher than that of the Negative Control.
- d) The absorbance of the 10 IU/ml Standard must be lower than that of the Low Positive Control.
- e) The absorbance of the 50 IU/ml Standard must be higher than that of the Low Positive Control.
- f) The Low Positive Control must be within its assigned range. This control is used to validate the low end of the assay.
- g) The High Positive Control must be >50 IU/ml. This control is used to validate the upper range of the assay.
- h) The Negative Control must be < 8 IU/ml. This control is used to validate the assay below the cut-off.

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

For Qualitative Assays

- a) The High Positive, Low Positive and Negative Controls must be included in each test run.
- b) The absorbance of the Blank must be < 0.2 .
- c) The Low Positive Control Index should be between 1.1-2.5.
- d) The High Positive Control Index should be > 2.5 .
- e) The Negative Control Index must be < 0.8 .

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

NOTES: The Negative and Positive Controls are intended to monitor substantial reagent failure. The controls will not control all parts of the procedure such as technical dilution of patient specimens. The Positive Controls will not ensure precision at the assay cut-off. Users may wish to establish an in-house control having a quantitative value determined by replicate testing, at or near the cut-off to monitor the precision of the assay cut-off. Additional controls may be tested according to guidelines or requirements of local, state or federal regulations or accrediting organizations.

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:

The Standards and Controls are provided ready to use: DO NOT DILUTE FURTHER.

Note: *For qualitative assays*, the 10 IU/ml Standard only is required. This Standard should be assayed in triplicate. In addition, a Blank (100 µl Sample Diluent only, in the first well of the first strip) is required and will be used to "zero" the photometer before reading test results.

For quantitative assays, all three Standards are required. No Blank is required; the 0 IU/ml Standard will function as the 'zero' and will be placed in the first well of the first strip. Standards can be run singly or in duplicate.

High Positive, Low Positive and Negative Controls must be run for either assay option.

1. Prepare 1:101 dilutions of the patient samples in Sample Diluent. (e.g., by addition of 2 µl sample to 200 µl Sample Diluent or 5 µl sample to 500 µl Sample Diluent)
2. Mix sample 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 Standards, controls and diluted patient sample, to the antigen wells. Avoid formation of bubbles when transferring diluted samples.
3. Allow the wells to incubate uncovered at 37 ± 3° C for 60 ± 5 minutes.
4. Aspirate or discard the contents of the wells. Remove any excess moisture in the wells by tapping on paper toweling. Wash the wells by rinsing 3 times with at least 300 µl 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 37 ± 3° C for 60 ± 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 37 ± 3° C for 20 ± 2 minutes.
10. Place 100 µl of Stop Solution into each well, avoiding bubble formation.
11. Read the absorbance of the wells at 450 nm using a reference wavelength of 600-630 nm. The plate should be read within 60 minutes of adding Stop Solution.

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

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

Qualitative Assay: Qualitative results may be obtained using the 10 IU/ml Standard only in triplicate, following a single Blank well (100 µl Sample Diluent only). If performing the qualitative assay option, manually set the reader for absorbance mode or cut-off control test mode and calculate the mean absorbance for the three Standard wells.

Note: When calculating the mean absorbance exclude any absorbance value that deviates by more than 15% from the mean absorbance value. Calculate the mean absorbance value from the two remaining absorbances. Exclusion of more than one of the 3 absorbance values invalidates the run.

*Example: Absorbance values obtained for 10 IU/ml Standard: 0.456, 0.445, 0.458 (after subtraction of the Blank)
Mean Absorbance of the 10 IU/ml Standard = 0.453
Sample Absorbance = 0.959
Index Values are then calculated as follows:
Sample Absorbance / Mean Absorbance of 10 IU/ml Standard = 2.13*

When using Diamedix Automated EIA Systems, results are automatically calculated and expressed as Positive, Equivocal or Negative.

Quantitative Assay : Quantitative results may be obtained from the point-to-point curve fit using all three Standards. For plate readers, the point-to-point option should be selected and Standard values entered accordingly. Index Values can be calculated by dividing the IU/ml values by 10 (the positive cut-off value). The Diamedix Automated EIA Systems will calculate results using the point-to-point curve fit and will print results automatically.

Specimens which yield absorbances greater than that of the 50 IU/ml Standard may be reported as 'greater than 50 IU/ml or Index > 5.0. Alternatively, such samples may be pre-diluted in Sample Diluent and retested. The resulting IU/ml or Index Value must be multiplied by the dilution factor for reporting.

Reference Ranges

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

Less than 8.0 IU/ml	Negative for Rubella IgG:
Index < 0.80	presumed non-immune.

Greater than/equal to 10.0 IU/ml Index \geq 1.0	Positive for Rubella IgG. presumed immune.
8.0 to 9.9 IU/ml Index 0.8-0.99	Equivocal*.

Note that when using the assay qualitatively the magnitude of the Index Value has no significance and results should be reported as under "Interpretation" above.

* When equivocal results are obtained, another specimen should be collected ten to fourteen days later and tested in parallel with the initial specimen. If the second sample is also equivocal, the patient is negative for primary or recent infection, and equivocal for antibody status. If the second sample is positive, the patient may be considered to have a primary infection. The conversion of an individual patient's serum from negative to positive for antibodies to the infectious agent in question, is defined as seroconversion, and indicates primary or recent infection.

Reporting Results

When the IU/ml value is reported for a single specimen the following statement should be included : "The following results were obtained with the Diamedix *Immunosimplicity* Is-Rubella IgG EIA Test System. The magnitude of the measured result, above the cut-off, is not indicative of the total amount of antibody present. The magnitude of the reported IgG level cannot be correlated to an end-point titer".

When the assay is used semi-quantitatively, the following statement should be included when reporting results: "Timing of specimen collection for paired sera may be critical. In some patients, antibody titers may rise to significant levels and fall again to lower or undetectable levels within a month. Other patients may not develop significant antibody levels. Culture results, serology and antigen detection methods should all be appropriately used along with clinical findings for diagnosis".

Paired Sera

To determine a significant difference between acute/convalescent sera, both specimens must be run within the same assay. In addition, paired sera should be evaluated within the reportable range of the assay. The upper limit of the reportable range has been set at 50 IU/ml. Studies by the manufacturer performed both manually and using the Diamedix Automated EIA Systems have shown that a 2.6-fold or greater increase in the IU/ml ratio(convalescent serum IU/ml value/ acute serum IU/ml value)corresponds to a four-fold increase in Rubella IgG antibody level and a 1.9-fold increase in the IU/ml ratio corresponds to a two-fold increase in Rubella IgG antibody level. Ratios in the range of 1.9 to less than 2.6 indicate an equivocal status for the paired samples. In this case, paired samples can be retested or additional samples collected if necessary. If paired sera controls are desired, it is recommended that a 1:4 dilution of a sample with an IU/ml value of between 40 and 50 be prepared in Sample Diluent. The undilute and 1:4 diluted material will provide a simulated serum pair. The Ratio of the undilute and 1:4 diluted material can then be compared against the established range.

Procedure Notes

1. Do not interchange reagents from different reagent lots except for Sample **A** Diluent, Wash **S** Concentrate, Substrate **HRP** and Stop **M** Solution.
2. Do not use reagents beyond their expiration date.
3. Store unused reagents at 2 to 8 °C.
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. Antigen coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.
7. (*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.*
8. The concentration of anti-Rubella IgG in a given specimen determined from assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

Limitations

1. The results obtained with the Is-Rubella IgG Test Kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves.
2. Rubella IgG ELISA assays are not intended to replace virus isolation and/or identification.
3. The prevalence of the analyte will affect the assay's predictive value.
4. Assay performance characteristics have not been established for visual result determination.
5. Performance of this assay has not been established on spectrophotometry utilizing a single wavelength.
6. The test should be performed on serum. The use of whole blood or plasma has not been established.
7. A single positive result only indicates previous immunologic exposure and cannot be used to distinguish between active and past infection. Paired samples (acute and convalescent) are required to detect seroconversion or a significant rise in antibody level.
8. A negative result does not always exclude the possibility of active rubella infection. The sample may have been collected before appearance of IgG antibody. If infection is suspected, a second sample should be collected at least 10 days after onset of rash and tested concurrently with the first sample to determine if seroconversion has occurred.

9. The performance characteristics have not been established for newborns using cord blood.
10. The results on serum from immunosuppressed individuals must be interpreted with caution.
11. The performance characteristics of the Diamedix Is-Rubella IgG Test Kit with automated equipment other than the Diamedix Automated EIA Systems have not been established.

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