**Rubella IgM
Capture Enzyme
Immunoassay**

Laboratory Name		
Adopted		
Reviewed		
Reviewed		
Revised		
Supercedes		

Method: **Diamedix Corp., Immunosimplicity®**
Manual or in conjunction with 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 lymphodenopathy. 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 fequently in vaccinated that 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. IgM antibodies are the first antibodies produced in response to rubella infection and become detectable 2-3 days after onset of symptoms. These antibodies reach peak levels by 14-21 days and then rapidly diminish in concentration over the next 4 to 8 weeks until antibody is no longer clinically detectable. The presence of IgM antibody in a single specimen suggests that the patient has recently experienced a rubella infection. IgG antibodies first appear several days after the IgM response, reach peak levels 14-21 days later, and then persist at varying levels for life (6,7).

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 for detecting IgG and IgM antibodies. Capture EIAs offer the additional advantage of avoiding interference due to rheumatoid factor and competing IgG antibodies.

The Diamedix Immunosimplicity® (Is)-Rubella IgM Capture Test Kit is a Capture EIA procedure intended for the qualitative detection of IgM antibodies to rubella antigen. The test can be performed either manually or in conjunction with one of the Diamedix Automated EIA Systems.

Principle of the Procedure

The Is-Rubella IgM Capture Test Kit utilizes ELISA based on the antibody-capture technique. Diluted patient sera are incubated with goat antibody against human IgM bound to the solid surface of a microtiter well. Patient IgM is 'captured' by the surface bound antibody. Unbound serum components are washed away. Patient anti-rubella IgM antibodies are 'detected' and bound by an immunocomplex, Enzyme Tracer, consisting of rubella antigen which is linked to a mouse monoclonal anti-rubella antibody conjugated to horseradish peroxidase. Unbound tracer 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 IgM 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 should 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. The NCCLS provides recommendations for collecting and storing blood specimens (8).

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

Reagents

Anti-IgM Coated Wells	Twelve, 8-well microwell breakapart strips, color-coded orange, coated with goat anti-human IgM.
Cut-Off Calibrator	One vial with blue cap containing 0.25 ml of human serum, preserved with 0.1% sodium azide, weakly reactive for rubella IgM antibodies. The Cut-Off Calibrator is used to determine the cut-off of the assay.
Low Positive Control	One vial with white cap containing 0.25 ml of human serum preserved with 0.1% sodium azide. Assigned range printed on label. The Low Positive Control is used to control the low range of the assay.
Negative Control	One vial with black cap containing 0.25 ml of human serum preserved with 0.1% sodium azide, non-reactive for rubella IgM antibodies. The Negative Control is used to control the negative range of the assay.

> *Note: The Cut-Off Calibrator and Controls are prepared from different serum lots.*

Lyophilized Antigen	Six vials of lyophilized rubella antigen, (grade IV sucrose purified, strain HPV 77 produced in Vero cells).
30X Tracer	One vial with red cap containing 0.8 ml mouse monoclonal anti-rubella antibody conjugated to horseradish peroxidase (30X concentrate) in stabilizer. The mouse monoclonal anti-rubella antibody is designated R22T and recognizes an antigen involved in hemagglutination.
Tracer Diluent	One bottle with red cap containing 25 ml borate buffer. Also includes protein stabilizers, gentamycin and Proclin™ 300 as preservatives. Color-coded pink.

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.
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.
Substrate G	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 $37 \pm 3°C$

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, Cut-Off Calibrator, controls and the materials that contact them as potential biohazards. Each donor unit in the Cut-Off Calibrator and controls has been found negative for Hepatitis B surface antigen, HCV and HIV-1 and 2 antibodies by FDA-approved third

generation tests. However, because no method can offer complete assurance that HIV-1 and 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. 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 horseradish peroxidase activity. Care must be taken to ensure that azide is not carried over from other reagents into the immunocomplex and substrate steps.

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 shown possible mutagenic effects in laboratory experiments.

Calibration

This test uses an in-house reference standard (Cut-Off Calibrator). The Cut-Off Calibrator has been derived from weakly positive sera and is titrated to an absorbance value equivalent to the cut-off of the assay. Samples whose absorbances exceed this value are considered positive for rubella IgM antibodies and samples whose absorbances are less than this value are considered negative for rubella IgM antibodies. To account for the inherent variations in enzyme immunoassays an equivocal range of $\pm$ 10% has been included at the assay cut-off.

Quality Control

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

b) The absorbance of the Blank must be < 0.100.

c) The absorbance of the Cut-Off Calibrator must be $\geq$ 0.150 when read against the blank.

d) The Low Positive and Negative Controls must be within their assigned ranges.

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

NOTES: Additional controls may be tested according to guidelines or requirements of local, state, and/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.

Each vial of lyophilized antigen is sufficient for at least 2 strips. Reconstitute only the number of vials required. Discard any unused Enzyme Tracer after the day's testing is completed.

MANUAL USERS:

1. Prepare 1:101 dilutions of the Cut-Off Calibrator (in triplicate), controls and 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 diluted Calibrator, controls and patient samples, to the wells. Avoid formation of bubbles when transferring diluted samples.

   Note: Include one well which contains 100 µl of Sample Diluent as a reagent blank. This will ultimately be used to "zero"the photometer before reading test results. DO NOT ADD ENZYME TRACER TO THE BLANK WELL.

3. Allow the wells to incubate uncovered at 37 $\pm$ 3°C for 60 ± 5 minutes.

4. As soon as the sample incubation has commenced, prepare the Enzyme Tracer by adding 2.9 ml of Tracer Diluent to each vial of lyophilized antigen required for the run. Mix until all the lyophilized material is reconstituted. Then add 100 µl 30X Tracer to each antigen vial and mix well. Allow the prepared Enzyme Tracer to sit at room temperature (18-30°C) for at least 30 minutes.

5. 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 each of Wash Solution. After the 3rd volume of Wash Solution, allow the wells to "soak" for at least one

minute prior to final aspiration/emptying. When using an automated washer, follow the manufacturer's instructions and set up the same wash procedure as described.

6. Place 100 µl of Enzyme Tracer into each well (except the blank), avoiding bubble formation.

7. Add 100 µl Sample Diluent to the Blank well.

8. Allow the wells to incubate uncovered at 37 $\pm$ 3°C for 60 ± 5 minutes.

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

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

11. Allow the wells to incubate uncovered at 37 $\pm$ 3°C for 20 ± 2 minutes.

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

13. Read the absorbance of the wells at 450 nm using a reference wavelength of 600-630 nm. The plate should be read 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

Calculate the MEAN absorbance of the Cut-Off Calibrator. Note: When calculating the mean absorbance of for the Cut-Off Calibrator exclude any absorbance value that deviates by more than 20% 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. Determine the Index Value for each patient sample or control using the following formula:

$$\frac{\textbf{Absorbance of Sample}}{\textbf{Mean Absorbance of Cut-Off Calibrator}} = \textbf{Index Value}$$

Example: Absorbance values obtained for Cut-Off Calibrator: 0.356, 0.345, 0.368 (after subtraction of the Blank)
Mean Absorbance of the Cut-Off Calibrator = 0.356
Sample Absorbance = 0.959
Index Values are then calculated as follows:
Sample Absorbance / Mean Absorbance of Cut-Off Calibrator = 2.69

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

Reference Ranges

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

Index Value	Interpretation
Index < 0.90	Negative for anti-rubella IgM.
Index $\geq$ 1.10	Positive for anti-rubella IgM.
Index 0.90-1.09	Equivocal for anti-rubella IgM.*

Note that 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.

Reporting Results

When reporting results the following statement should be included: "The following results were obtained with the Diamedix Immunosimplicity Is-Rubella IgM Capture EIA Test Kit. The magnitude of the measured result, above the cut-off, is not indicative of the total amount of antibody present."

Procedure Notes

1. The reported concentrations of anti-rubella IgM in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

2. Do not interchange reagents from different reagent lots except for Sample **A** Diluent, Wash **S** Concentrate, Substrate **G** and Stop **M** Solution.

3. Do not use reagents beyond their expiration date.

4. Store unused reagents at 2 to 8° C.

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

6. The Capture ELISA 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.

7. Coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.

8. *(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. The results obtained with the Is-Rubella IgM Capture Test Kit serve only as an aid to diagnosis of current or recent infection. They should be interpreted in conjunction with other clinical findings and diagnostic procedures.

2. Rubella IgM ELISA assays are not intended to replace virus isolation and/or identification.

3. Rubella vaccination usually results in elevated levels of specific IgM antibodies which may persist for several months.

4. Low levels of IgM antibodies may occasionally persist for more than 12 months post-infection. This response may be distinguished from the early IgM response to infection by testing samples two weeks later to ascertain if there is a change in IgM antibody level.

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

6. Performance of this assay has not been established on spectrophotometry utilizing a single wavelength.

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

8. A negative result does not always exclude the possibility of active rubella infection. The sample may have been collected before the appearance of IgM antibody. If infection is suspected, a second sample should be collected at least 10 days after onset of rash and tested.

9. An IgM response can sometimes accompany re-infections.

10. The performance characteristics have not been established for newborns using cord blood.

11. The results on serum from immunosuppressed individuals must be interpreted with caution.

12. The performance characteristics of the Diamedix Is-Rubella IgM Capture Test Kit with automated equipment other than Diamedix Automated EIA Systems have not been established.

References

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4. Mitchell, L.A., Ho, M.K.L., Rogers, J., Tingle, A. J., Marusyk, R.G., Weber, J. M., Duclos, P. Tepper, M.L.,Lacroix, M. and Zrein, M. 1996. Rubella Reimmunization: Comparative Analysis of the Immunoglobulin G Response to Rubella Virus Vaccine in Previously Seronegative and Seropositive Individuals. J. Clin. Microbiol. 34: 2210-2218.

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8. Procedures for the Handling and Processing of Blood Specimens: Approved Guideline- Second Edition NCCLS Document H18-A2, Vol. 19, No. 21.1999.

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