1. Purpose
This document establishes the procedures for performing a rubella IgM ELISA using the Serion ELISA classic Rubella Virus IgM commercial test kit. This assay is used for the detection of rubella specific IgM antibodies in patient serum or plasma. Detection of specific IgM antibodies in a serum sample collected within the first few days after rash onset can provide presumptive evidence of a current or recent rubella virus infection. The optimum time-point for collection of serum is five days after the onset of symptoms (fever and rash) when >90% of cases will be IgM positive. On the day of rash onset only about 50% of cases are IgM positive. Therefore, if serum collected less than five days after onset is negative, a second sample would be necessary to confirm/rule out rubella.
When a patient with suspected rubella has been recently vaccinated (<30 days prior to blood draw) IgM cannot distinguish rubella disease from the response to vaccination. Determination of the rubella genotype is necessary when rubella symptoms occur following an exposure to wild type virus and rubella containing vaccine has been provided as post-exposure prophylaxis.

2. Background and Test Principle
Rubella virus is a human pathogen belonging to the Togavirus family of RNA viruses. The virus is transmitted by respiratory secretions and causes a mild disease characterized by rash and fever. Rubella infection during pregnancy may lead to severe damage to the fetus, a condition known as congenital rubella syndrome. To detect rubella IgM antibodies in a serum sample, microtiter wells are coated with rubella antigen. The sample is added to the well and rubella-reactive antibodies will bind to the bound antigen. After the removal of unbound material, rubella-reactive IgM antibodies in the sample can be detected using a commercial anti-human IgM antibody that is conjugated to an enzyme (alkaline phosphatase). After washing to remove unbound conjugate, a substrate (para-nitrophenylphosphate) is added. This reacts with the conjugate to produce a color change. The color intensity can be quantified photometrically and is proportional to the level of bound IgM antibody.

3. Related Documents
- Serion Activity Master Worksheet or Serion IgM Results Worksheet QC
- Serion Job Aid

4. Equipment
- Incubator 37°C
- Vortex
- Absorbance Microplate Reader
- Automated Microplate Washer (optional)
- Freezer -20°C
- Refrigerator, 4°C

5. Reagents and Media
- In-house positive serum control, stored at -20°C
- In-house negative serum control, stored at -20°C
• Serion RF-Absorbent (Catalog #T 200, 20ml)
• Serion Rubella Virus IgM Kit (Catalog # ESR 129M)
  ▪ Microtiter test strips (MTP)
  ▪ Standard serum (ready to use, STD)
  ▪ Negative Control serum (ready to use, NEG)
  ▪ Anti-human IgM conjugate (ready to use, APC)
  ▪ Washing solution (WASH)
  ▪ Dilution buffer (DILB)
  ▪ Stopping solution (STOP)
  ▪ Substrate (ready to use, pNPP)
  ▪ Quality control certificate

6. Supplies, Other Materials
   • PPE (lab coat, gloves, goggles)
   • Test sera
   • Distilled or deionized water
   • Microcentrifuge tubes, titer tubes, or deep well plates
   • Squeeze bottle (if not using automated washer)
   • Micropipetters 1-20μl, 20-200μl, and 200-1000μl
   • Multichannel pipetter 50-300μl or 20-200μl (optional)
   • Pipette tips
   • Pipette aide
   • Serological pipettes 5ml, 10ml, 25ml
   • Thermometer for room temperature
   • Paper towels
   • Reagent reservoirs
   • Moist chamber
   • 10% bleach solution
   • Container to discard waste
   • Tape

7. Safety Precautions
   • Follow universal precautions while working with blood and/or blood products.
   • Wash hands before and after handling reagents. Cross-contamination of reagents and/or samples could cause erroneous results.
   • Never pipette by mouth or allow reagents or patient sample to come into contact with skin.
   • Handle all blood and serum as if capable of transmitting disease.

8. Sample Information / Processing
   • Store serum between 2-8°C if testing will take place within 1 week. If specimens are to be kept for longer periods, store at -20°C or colder.
   • Do not use a frost-free freezer because it may allow the specimens to go through freeze-thaw cycles and can degrade antibody.
   • Samples that are improperly stored may yield erroneous results.
   • Lipaemic, hemolytic, or icteric samples should only be tested with reservations.
   • Obviously contaminated samples should not be tested due to the risk of incorrect results.
   • If paired sera are to be collected, acute samples should be collected as soon as possible after the onset of symptoms. The second sample should be collected 14 to 21 days after the acute specimen was collected. Both samples must be run on the same plate.
9. **Quality Control**

- Use only distilled or deionized water and clean glassware.
- Prepare working dilutions of the assay reagents identically each time the assay is run.
- Strictly observe incubation times and temperatures.
- Match patient identifiers with serum dilution tubes and location of sera on test plates with calculated OD values.
- A record must be kept of each assay as the test is performed. Note the reagents, lot numbers, and expiration dates. Record how dilutions are made and the timing of each step.
- Record and monitor control values for in-house positive and negative sera controls in order to review assay performance over time.
- Do not use kits after the expiration date.

A. **Controls**

- Include a substrate blank, standard serum, and negative controls on each plate in each run.
- Run the standard serum in duplicate.
- Run in-house positive and negative sera controls with every assay, in addition to the controls that are included in the kit.

B. **Accepted Values**

- The variation of OD-values may not be higher than 20% for the standard control sera.
- The OD of the substrate blank must be $<0.25$.
- The mean OD value of the standard sera control after subtraction of the substrate blank must be within the validity range given on the lot specific quality control certificate in the kit.
- The negative control must be negative.

C. Repeat the assay if quality control requirements are not met. If the assay fails QC again on the retest, discuss next course of action with supervisor.
10. Test Procedure (refer to kit insert)

- Bring all reagents to room temperature (20-25°C). Use a calibrated thermometer to verify that room temperature is within the specified range.
- Prepare the Serion Activity sheet or Serion IgM worksheet and Job Aid. Calculate the number of wells needed for the assay including the blank, positive, negative, and in-house controls (IHC).
- Using the gray section on the worksheet, determine the amount of working stock of RF-DILB buffer needed and the amounts of both RF-absorbent and dilution buffer to make a 1:5 solution. Determine the amount of working stock Wash buffer is needed and the amount of concentrated Wash and distilled water needed to make a 1:30 dilution.
- Samples must be diluted prior to use. Do NOT dilute the negative control and standard sera with RF-DILB buffer. They are ready to use.
- Dilute the RF-absorbent 1:5 with dilution buffer following the calculations on the worksheet. (i.e: for each test sample, add 200 µl of RF-absorbent to 800 µl of dilution buffer)
- Vortex the test samples and controls prior to use.
- Dispense the 1000 µl RF-DILB solution into micro/titer tubes or deep well plate, one for each sample to be tested, including in-house controls (IHC).
- Dilute the test samples 1:101 with the RF-dilution buffer mixture, being sure to switch tips between each sample. (i.e: add 10 µl of test sample to 1000 µl RF-dilution buffer mixture) Mix well by using a vortex or repeatedly pipetting up and down at least 10 times. Do not aerosolize.
- Incubate the diluted samples at room temperature (20-25°C) for 15 minutes (minimum). Samples mixed with RF-dilution buffer can be stored in sealed vials for up to one week at 2-8°C. If using refrigerated samples, bring to room temperature before proceeding.
- Do NOT dilute the negative control and standard sera with the RF-dilution buffer mixture. They are ready-to-use.
- Dilute the washing buffer (WASH) concentrate 1:30 with distilled water following the calculations on the worksheet. (e.g: add 10 ml of buffer to 290 ml of water for a final volume of 300 ml; 1 full plate will require 240 ml) Buffer can be stored for up to two weeks at 2-8°C. If using refrigerated buffer, bring to room temperature before proceeding.
- Open the bag containing the microtest strips and remove any extra strips from the frame. Tape test strips into the frame to prevent them from ejecting during the wash processes. Replace the extra strips and the desiccant into the bag, seal with tape and return to refrigerator.
- Add 100 ul of diluted samples or ready-to-use controls into the appropriate wells, changing tips for each sample, as follows:
  - Well A1 substrate blank (leave empty)
  - Well B1 kit negative control (NEG)
  - Well C1 kit standard serum (STD1) <positive control>
  - Well D1 kit standard serum (STD2) <positive control>
  - Well E1 in-house positive serum 1
  - Well F1 in-house negative serum 2
  - Well G1 test serum 3...
- Incubate the test plate for 60 minutes (+/- 5 min) at 37°C (+/- 1°C) in a moist chamber (e.g. a
sealed plastic bag containing a damp paper towel)

- After the incubation is complete wash all wells with diluted washing solution as follows:
  - Aspirate or shake out the incubation solution (dump into container and treat with 10% bleach solution)
  - Fill each well with 300 ul washing solution
  - Aspirate or shake out the washing solution
  - Repeat the washing procedure 3 more times (total 4)
  - Blot any remaining liquid on a paper towel
- Add 100 ul of conjugate (APC) to each well (except the substrate blank) and incubate for 30 minutes (+/- 1 minute) at 37°C in a moist chamber.
- Repeat washing step as above.
- Add 100 ul of substrate solution (pNPP) to each well including the well for substrate blank, and incubate for 30 minutes (+/- 1 minute) at 37°C in a moist chamber.
- Add 100 ul stopping solution (STOP) to each well.
- Read optical density within 60 minutes at 405 nm, reference wavelength between 620 nm and 690 nm (e.g. 650 nm).

11. Manual Calculations to determine assay validity and interpretations
- The Serion manual validation worksheet can be used to manually calculate results.
- Fill out the upper portion of the worksheet with pertinent information.
- Enter the raw OD values in column B.
- Determine the Blank Reduced OD of all kit and in-house controls by subtracting the raw OD of the Blank from the Raw OD of kit and in-house controls.
- Determine the validity of the assay by answering the questions listed. If this section indicates all data pass, the assay is valid and results can be reported.
- Determine all sample blank reduced OD values.
- Based on the mean of the blank adjusted standards, use the QC chart, included with the kit, to determine the cut off values for interpreting results as either positive, borderline, or negative.

12. Calculations using the Serion Activity sheet

Calculations and quality control can be performed using the Serion Activity Excel Worksheet, which can be obtained upon request from Serion or distributors of Serion after purchase of a kit. The current version is V12.0. Alternately, an Excel worksheet can be created using the criteria and formulas given in the SERION instruction manual.

Enter data into Sheet 1. Sheet 2 of the Activity worksheet provides instructions for filling out the worksheet and evaluating whether a test has passed quality control.

- At the top of Sheet 1 of the worksheet, there are 8 blocks for tests: Test 1 through Test 8. Only one block needs to be completed per kit (e.g. Test 1=Rubella IgM kit data, Test 2=Measles IgM kit data).
- Using the Quality Control certificate that comes in the Serion kit, fill out the required information in the Test block. The Sheet 2 instructions identify where the required information can be found on the certificate using numbers that correspond to the required information (e.g. #3 is the Kit Lot, #4 is the lower borderline value).
- Next, enter OD data from the test run in the second set of blocks (directly below the Test set).
This includes the OD values for the two standards. The % deviation (Δ %) for the standards will be automatically calculated. If the blank reduced data has already been subtracted, enter 0 in the Blank cell (BLK). If ODs have not had the blank subtracted, enter the OD from the substrate blank well in the Blank cell.

- In the bottom set of blocks, first enter the Test number at the top of the block (e.g. if the Test 1 block was filled out with information from the measles IgM kit, enter 1 in the cell at top of the lower block. Depending on the number of samples, more than one block may need to be used.
- In the first block, enter STD in the A1 ID column and the mean value (MV) of the standard (as determined in row 17) in the A1 OD column. Next, enter NC in the A2 ID column and the OD value of the negative control in A2 OD column. For the test to be valid the units (U/ml) for the standard should exactly match the reference value given on the certificate (#11) and the negative control must yield a negative result.
- If the above conditions are met, enter the ID numbers and OD values for each test sample in the block(s). If 0 was entered in the Blank cell, enter blank reduced ODs. If the OD for the blank was entered in the Blank cell, enter measurement ODs. Press the enter button.
- The antibody activity is calculated and presented in the Units column while the interpretation is presented in the Int. column.
- Save the completed sheet under a new name and save along with the raw data.

### 13. Calculations using the Serion IgM results worksheet

- The Serion IgM results worksheet can be used to prepare the plate layout as well as for calculating results.
- Fill out the upper portion of the worksheet with pertinent information.
- At the bottom of the page is a template to prepare the plate layout.
- Using this information, determine the total number of wells being used on the plate. Enter this data into the indicated well for this information in the either the cell for using a plate washer or the cell if manually washing.
- Subtract 4 from the total number of samples and enter into cell for RF/DILB information contained in the gray box. It will automatically calculate the amount of RF/DILB solution and Wash buffer to make to process the number of samples on the plate.
- Once raw OD values have been obtained, enter them into the indicated bottom section for raw OD values.
- Using the Quality Control Certificate from the kit, fill in the value for the validity range lower limit in the purple box, and validity range upper limit in the pink box.
- Locate the value of the Standard Controls Mean OD which is automatically calculated and displayed in the orange box at the top of the page. Find which range this value fits into on the QC certificate. This section will indicate the cut-off values for results interpretation.
- Enter the Lower Cut-off OD in the green box and the Upper Cut-off OD in the blue box at the top of the page, which are determined by the appropriate section of the QC certificate, located in the above step.
- Determine the validity of the assay by verifying the information contained in the red box under Quality Control. If this section indicates all data pass, the assay is valid and results can be reported.
- The column Final OD automatically subtracts the Blank OD value from all wells.
- Based on the QC data entered from the kit and the Blank subtracted OD values, a qualitative result appears in the Result column as either positive (Pos), borderline (B), or negative (Neg).
14. Interpretation

Use U/ml for the Borderline range given on the Quality Control Certificate
- Samples with U/ml less than the lower limit of the borderline range are negative.
- Samples with U/ml greater than or equal to the lower limit of the borderline range and less than or equal to the upper limit of the borderline range are equivocal.
- Samples with U/ml greater than the upper limit of the borderline range are positive.
- The limits of quantification are given on the Quality Control Certificate. If positive test result units are above the upper limit of quantification the sample must be analyzed at a higher dilution to obtain accurate quantification. The resulting antibody activity must be multiplied by the additional dilution factor to obtain the correct antibody activity of the sample.
- Undiluted samples with units above the upper limit of quantification should be reported as >upper limit (e.g. if the upper limit is given as 120 U/ml and the undiluted sample measures as 400 U/ml, the reported result should be >120 U/ml).
- Undiluted samples with units below the lower limit of quantification should be reported as <lower limit (e.g. if the lower limit is given as 5 U/ml and the undiluted sample measures as 2 U/ml, the reported result should be <5 U/ml).

15. In-House Controls

- Each run of ELISA test must include both a positive and negative IHC serum.
- If the negative IHC serum is positive, the run needs to be repeated.
- The positive IHC serum needs to be tracked using a Levey-Jennings chart. Each positive IHC OD value is plotted to give a visual indication of the stability of the method over time. The chart determines the number of standard deviations (SD) the actual result is from the mean. Ideally the IHC should not exceed 3 SD.