

Laboratory Procedure Manual

Analyte: **Measles, Rubella, and Varicella-Zoster
Antibodies**

Matrix: **Serum**

Method: **Enzyme Immunoassay (EIA)**

Method No.:

Revised:

as performed by: *California State Department of Health Services
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Important Information for Users

California State Department of Health Services periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released analytes follows:

| Lab Number | Analyte | SAS Label |
|-------------------|----------------|--------------------------------|
| l19_c | LBDRUIU | Rubella in international units |
| | LBXME | Measles antibodies |
| | LBXVAR | Varicella-Zoster antibodies |

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

To better define the prevalence of antibody to measles virus, rubella virus, and varicella-zoster virus (VZV) in the U.S. population, a subset of the serum samples collected during the NHANES 1999+ will be tested by enzyme immunoassay (EIA).

These EIA tests have been developed by the staff of the Immunoserology Unit of the California State Department of Health Services (CSDHS) Viral and Rickettsial Disease Laboratory (VRDL). The procedures described below are the standardized protocols of the VRDL's in-house EIA tests for serodiagnosis of viral infections and are currently routinely used for the following viruses: adeno, cytomegalo, herpes simplex, influenza A and B, measles, mumps, rubella, parvo-B19, respiratory syncytial, St. Louis encephalitis, varicella-zoster, and western encephalitis. The individual steps in the test are the same for all these viruses, except that production and purification of viral and control antigens used in the assay are different for individual viruses. These assays are approved and routinely monitored by Clinical Laboratory Improvement Amendments (CLIA) staff.

In the indirect EIA, a suitable antigen material (i.e., solubilized varicella-zoster virus) is coated on the wells of a 96-well microtiter plate, which is subsequently incubated with a diluted test specimen. If the specimen contains antibody to the antigen, the antibody will form complexes with the antigen on the coated plate. After washing unreacted serum components from the plate, an antibody-enzyme conjugate is added to the wells and incubated. The conjugate consists of anti-human IgG covalently coupled to the enzyme alkaline phosphatase. The conjugate will react with the antigen-antibody complex on the surface of the well resulting in a sandwich of well-antigen-antibody-antibody-enzyme. If the test specimen does not contain IgG antibody to the antigen, the conjugate will not bind to the well surface and will be removed by washing. The presence of enzyme in the complex is determined by adding an enzyme substrate (indicator system) to the well and incubating while a color reaction occurs. The enzyme substrate reaction will result in a yellow colored product which is measured in a spectrophotometer adjusted to a wavelength of 405 nanometers with a side band adjusted to 630 nanometers.

Measles virus causes an acute, generalized infection that until recently has been one of the most common viral diseases of childhood worldwide. Measles is rarely fatal in North America or Europe, but mortality after measles virus infection can be as high as 20% in developing countries. Measles is spread from an infected person to a susceptible person mainly via aerosol. Worldwide, there are 70 million cases of measles each year, resulting in more than a million infant deaths.

Rubella (German measles) is generally a mild viral disease. However, if contracted during the first trimester of pregnancy, the virus may produce a severe infection in the fetus resulting in multiple abnormalities referred to as congenital rubella syndrome. Additional consequences of rubella infection may include spontaneous abortion of the fetus, miscarriage, or stillbirth. Women of childbearing age infected with rubella virus represent a significant public health problem because of the fetal congenital defects associated with this disease.

The level of antibody providing resistance to infection with the rubella virus has been established using hemagglutination inhibition (HAI) procedures. A publication from the Centers for Disease Control and Prevention states that "any level of detectable antibody should be considered presumptive evidence of immunity." Field evaluations have shown that the VRDL EIA test for rubella results are equivalent to HAI results in determinations of immune status.

Varicella-zoster virus is the etiologic agent of chickenpox (varicella) and shingles (zoster). The major role of serological diagnosis of varicella-zoster virus antibodies is to determine the immune status of susceptible individuals in high risk groups such as immunocompromised patients, hospital employees, transplant recipients and pregnant women.

2. SPECIAL SAFETY PRECAUTIONS:

Wear gloves, lab coat, and safety glasses while handling all human blood products and infectious virus. Disposable plastic, glass, and paper (pipet tips, reaction wells, gloves, etc.) that contacts blood or virus is to be placed in a biohazard autoclave bag or discard pan to be autoclaved. Use disposable plastic backed paper for all work surfaces. Viral antigens which have been inactivated should be treated as if they are infectious.

Material Safety Data Sheets (MSDS) and lists of hazardous chemicals in use in this procedure are kept in room 2003.

The Laboratory Safety Guidelines of the Viral and Rickettsial Disease Laboratory are followed by all personnel in the laboratory.

2. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

A. The data on the NHANES Shipping Manifest disks are read into the MS ACCESS database and specimen listings are checked for duplicate, inconsistent, or missing data. Run sheets are generated corresponding to specimen location and read into EXCEL spreadsheet files.

B. The data that is captured from the microplate reader using LABTRONICS COLLECT software is merged with corresponding runsheets in EXCEL and read into MS ACCESS.

C. The final form of data will be in a comma delimited text format as required.

D. Routine backup procedures include: 1) daily backup of each day's test data on hard disk; and 2) weekly backup of data on 100MB ZIP disk.

3. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

A. Type of specimen

(1) Serum specimens. Collect and separate specimens aseptically. Store at 4–8°C or –70°C. Plasma specimens or very hemolyzed specimens are not recommended as they may give unreliable results. Specimens containing cellular debris or red blood cells should be clarified prior to use. Specimens with microbial contamination should not be used.

B. Time of collection

(1) To test for immunity status a single specimen is sufficient. For immunity status after exposure, the specimen must be collected within 7 days of the exposure.

(2) To test for acute or recurrent infection, both acute-phase and convalescent-phase specimens are required. The acute-phase specimen should be collected within 7 days of onset of symptoms and the convalescent-phase specimen 14 days or more after onset of symptoms.

C. Storage

(1) Specimen stability has been demonstrated for 1 year at –20°C and indefinitely at –70°C.

(2) Once received, the specimens remain frozen at ≤ –70°C until time for analysis. Samples are thawed prior to testing and vortexed to ensure thorough mixing. The samples remain at 4–8°C until testing is complete, then are refrozen at ≤ –70°C. Samples thawed and refrozen several times are not compromised.

4. PREPARATION FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure

5. PREPARATION OF ANTIGENS, REAGENTS, CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

A. PREPARATION OF MEASLES VIRUS EIA ANTIGEN

- (1) Prepare virus infected monolayers by mixing measles virus infected human fetal diploid lung (HF DL) cells with uninfected HF DL cells at a ratio of 1:3.
- (2) Harvest cell monolayer when the cells show 3–4+ CPE (usually 7-10 days).
- (3) Dislodge the cell monolayer with sterile glass beads into medium.
- (4) Centrifuge the medium and cells at $1,000 \times g$ for 10 min. Save supernatant and pellet.
- (5) Freeze/thaw the pellet in ethylene glycol and dry ice (3x).
- (6) Take supernatant collected and centrifuge at $78,000 \times g$ for 1.5 h. Remove supernatant and discard.
- (7) Resuspend pellets from all tubes, including freeze/thaw pellet, in EIA solubilizing buffer to solubilize and inactivate the virus.
- (8) Sonicate the solubilized antigen at 10–15 sec intervals (90 sec) to dissociate viral aggregates.
- (9) Centrifuge $2,000 \times g$ for 10 min. Collect clarified supernatant.
- (10) Prepare uninfected control antigen (uninfected HF DL) in the same manner as the infected antigen.
- (11) Titrate the measles and control antigen in 96-well microplates to determine which dilution will be used in test proper. For the present study a dilution of 1:500 for measles and control antigen was determined.
- (12) Store at -70°C in volumes suitable for coating one batch of 20 plates to avoid repeated freezing and thawing of antigen.

B. PREPARATION OF RUBELLA EIA ANTIGEN

- (1) Trypsinize BHK-21 cells and infect with rubella virus (RV strain) with a multiplicity of at least 1 plaque forming unit (pfu) per cell in suspension. Incubate for one h with gentle shaking.
- (2) Plant approximately $7-8 \times 10^6$ cells per T-150 bottle with growth medium containing 10% fetal bovine serum (FBS).
- (3) After 24 h post infection, replace growth medium with maintenance medium containing 2% inactivated FBS.
- (4) After additional 48 h incubation, remove and reserve medium from the cell monolayer. Replace with fresh maintenance medium.
- (5) Clarify medium by centrifuging at $2,000 \times g$ for 30 min at 4°C .
- (6) Centrifuge the supernatant to pellet the virion at $78,000 \times g$ for 2 h. Discard the supernatant.

- (7) Repeat the harvest (steps 4–6) 2 times after 24-h incubation.
- (8) Resuspend all pellets in 1/50 to 1/60 of the original volume, depending on the pellet sizes, in EIA solubilizing buffer to solubilize and inactivate the virus.
- (9) Sonicate the solubilized antigen at 10–15 sec intervals (90 sec) to dissociate viral aggregates.
- (10) Prepare uninfected control antigen (uninfected BHK-21 cells) in the same manner as with the infected antigen.
- (11) Titrate the RV and control antigen in the 96-well microtiter plate to determine which dilution to be used in the test proper. For the present study a dilution of 1:100 for RV and control antigen was determined.
- (12) Store at -70°C in volumes suitable for coating one batch of 20 plates to avoid repeated freezing and thawing of antigen.

C. PREPARATION OF VARICELLA-ZOSTER VIRUS EIA ANTIGEN

- (1) Prepare virus infected monolayers by mixing VZV infected human fetal diploid lung (HFDL) cells with uninfected HFDL cells at a ratio of 1:3.
- (2) Harvest cell monolayer when the cells show 3-4+ CPE (usually 72-96 h).
- (3) Dislodge the cell monolayer with sterile glass beads into medium.
- (4) Centrifuge the medium and cells at $1,000 \times g$ for 10 min. Save supernatant and pellet.
- (5) Freeze/thaw the pellet in ethylene glycol and dry ice (3x).
- (6) Take supernatant collected and centrifuge at $78,000 \times g$ for 1 h. Remove supernatant and discard.
- (7) Resuspend pellets from all tubes, including freeze/thaw pellet, in EIA solubilizing buffer to solubilize and inactivate the virus.
- (8) Sonicate the solubilized antigen at 10–15 sec intervals (90 sec) to dissociate viral aggregates.
- (9) Prepare uninfected control antigen (uninfected HFDL) in the same manner as the infected antigen.
- (10) Titrate the VZV and control antigen in 96-well microplates to determine which dilution will be used in test proper. For the present study a dilution of 1:800 for VZV and control antigen was determined.
- (11) Store at -70°C in volumes suitable for coating one batch of 20 plates to avoid repeated freezing and thawing of antigen.

D. COATING OF VIRAL ANTIGEN AND CONTROL ANTIGEN TO 96-WELL MICROPLATE

- (1) Important: All new lots of viral and control antigen must be titrated for optimal dilution before being used for coating.
- (2) Color code microplates prior to coating with antigen.
 - (a) (Example: blue, Measles; red, Rubella; yellow, VZV).

- (3) Add 100 μ L of the working dilution of viral antigen and control antigen to each 96-well plate (control antigen in odd strips/viral antigen in even strips). Dry the microplate 3–5 h at 37°C with a fan blowing an air stream over the surface.
- (4) Store microplates as follows. Package each dried microplate in a heat-sealed plastic bag. Include a desiccant and humidity indicator in each bag. Store the plates at 4°C. The coated microplates are stable for at least 6 months. Do not use them if the humidity indicator has changed (blue to red).

E. STOCK REAGENTS

Unless otherwise specified, all chemicals are American Chemical Society "Reagent Grade". Reagents may be prepared with tap-distilled water except where glass-distilled water is specified.

- (1) Phosphate buffered saline (PBS), 10 \times stock, pH 7.2–7.4

Sodium chloride (NaCl).....85.0 gm (EM Science SX0420-1)
Disodium phosphate (Na_2HPO_4)....5.65 gm (Sigma S-9390)
Potassium phosphate (KH_2PO_4).....1.35 gm (Sigma P-3786)
Distilled water to.....1000 mL

Store the solution at room temp for no longer than a month. Discard the solution if it becomes contaminated.

- (2) Sodium azide, 10%

Sodium azide (NaN_3).....10.0 gm (EM Science SX0299-1)
Distilled water to.....100 mL

Use gloves when weighing azide. Do not inhale dust. Clarify the solution if necessary, using a 0.45 μ m filter. Store the solution at room temp. When discarding solutions with azide, autoclave them to break down the azide. If autoclaving is not possible, pour them down the sink followed by a large volume of water. Store the solution indefinitely.

- (3) Tween 20, 10%

Tween 20.....10 mL (Sigma P-1379)
10% sodium azide.....1 mL
Distilled water to.....100 mL

Store solution at room temp for no more that six mo.

- (4) Magnesium chloride solution, 0.5 M with 20% sodium azide

Sodium azide.....20.0 gm (EM Science SX0299-1)
Magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$).10.2 gm (Baker 2444)
Distilled water to.....100 mL

See step (2) above for preparation of sodium azide. Store the solution at room temp indefinitely.

- (5) 1 N HCl

HCl (12 N) conc.50 mL (EM Science HX0603-3)
Glass distilled water.....500 mL

Add HCl to water. Store solution at room temp for no more than 1 week.

(6) Diethanolamine buffer, pH 9.8 (DEA)

Diethanolamine (mw = 105.14) $\text{HN}(\text{CH}_2\text{CH}_2\text{OH})_2$,
AKA: 2,2'-iminodiethanol.....340 mL (Sigma D-8885)
Glass distilled water.....2400 mL
Sodium azide stock containing 0.5M
magnesium chloride.....3.5 mL
Glass distilled water.q.s.....3500 mL

In a 4-L beaker, add 1 L of glass-distilled water and begin stirring. Add 340 mL of diethanolamine. Rinse graduated cylinder with 1 L of glass-distilled water and add to beaker. Add another 400 mL of glass-distilled water. While stirring, adjust to pH 9.8 with 1 N HCl (approximately 300 to 600 mL 1 N HCl are needed). Record volume of 1N HCl used. Add 3.5 mL of stock solution containing 0.5 M magnesium chloride and 20% sodium azide. QS to 3.5 Ls and check final pH for 9.8; adjust if necessary. Store DEA at 4°C for no more than 12 months.

(7) Paranitrophenyl phosphate substrate (PNPP) (Sigma Chemical Co. #104)

Obtain PNPP as pre-weighed capsules in amounts suitable for one run. Store PNPP at -20°C or lower. Discard if color has changed from cream colored to yellow. (Tablets may be used, but take longer to dissolve)

(8) Conjugate – IgG (Sigma Chemical Co. Product #A-5403 or equivalent)

Anti-human IgG (gamma chain specific) $\text{F}(\text{ab}')_2$ fragment labeled with alkaline phosphatase. Store at 4°C until expiration date given by manufacturer. Titrate each lot to determine use dilution.

(9) Enzyme stopping reagent – trisodium phosphate (TSP) ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$)

TSP.....20.0 gm (Baker 3836-01)
Distilled water.....1000 mL

Store in hard glass bottle at room temp for no longer than 12 months.

(10) Solubilizing buffer – Tris/saline/EDTA solubilizing buffer, pH 9.0, for virus solubilization and inactivation.

Tris(HCl).....1.21 gm (Sigma T-3253)
EDTA.....0.83 gm (EM Science EX0550-5)
Disodium deoxycholate.....1.00 gm (Matheson DX 115)
NaCl.....8.00 gm (EM Science SX0420-1)
Nonidet P-40 (NP-40).....1 mL/L (Particle Data Lab NP-40)

Adjust pH to 9.0, QS to 1 L using glass-distilled H_2O .
Add 1% Aprotinin (Sigma A-6279) as protease inhibitor. Sterilize by membrane filter 0.22 μm . Store at 4°C.

(11) Casein in phosphate-buffered saline:

specimen diluent stock (blue)
conjugate diluent stock (red)

Tare a 4-L beaker and add to it:
Casein (Hammersten quality).....40.0 gm (Research Organics 1082C-1)
*Sodium azide.....8.0 gm (EM Science SX0299-1)

(*Be careful not to inhale dust)

Glass-distilled water.....3600 mL
10x PBS.....400 mL
10% Tween-20.....20 mL

Stir for 1 h. Adjust pH with 1 N NaOH to 7.40. QS with glass-distilled water to 4L. Stir at room temperature until cooled to 37°C. Pour casein solution into 400 ml bottles. Add 40 µL of Schilling food coloring (red or blue) to each 400 mL. Store at 4°C for no longer than 6 mo.

(12) 5% Bovine Albumin (BSA) in phosphate buffered saline (PBS)

- 10x PBS.....10 mL
- Glass Distilled Water.....89 mL
- Bovine Albumin (Armour Fraction V)....5 gm (Spectrum AL135)
- Sodium Azide 10%1 mL
- Mix ingredients in order given. Stir until BSA is dissolved. Filter using a 0.2 µm filter. Store at 4°C indefinitely.

F. WORKING REAGENTS

(1) Phosphate buffered saline (PBS), 1×

Dilute the 10x phosphate buffered saline stock solution 1:10 in distilled water. Store the solution at room temperature. Prepare fresh as needed. Store at room temperature.

(2) Phosphate buffered saline - Tween 20 (PBS-T)

Add 5.0 mL of the 10% Tween 20 solution per L of PBS and mix well. Working solution may be used for two weeks. Store at room temperature. PBS-T is used for washing plates.

(3) Serum diluent

Use 1× casein blue in PBS-T.

600 µL is needed for each patient serum and control sera. Store at 4°C for no longer than 6 months.

(4) Viral antigen and control antigen

Use viral antigens and matching non-infected cell control antigens prepared for EIA. The non-infected cell antigen is to be used as the non-specific antigen control. Store the antigens at -70°C. Aseptic techniques must be adhered to for the preparation and growth of viral antigen.

(5) Conjugate diluent

Use 1× casein red in PBS-T. Warm to room temperature before use.

(6) Working conjugate dilution

Dilute conjugate in red conjugate diluent. Use the dilution previously determined by titration. Prepare no sooner than 60 min prior to use.

(7) Substrate

Warm to room temp enough DEA buffer for the day's run. Add 1 mg PNPP substrate per mL of DEA buffer used. Pour in pre-weighed powder from capsule. Discard empty capsule. Prepare no sooner than 60 min prior to use.

G. CONTROL SERA

(1) Rubella Standard

Obtain WHO or CDC 1000 International Unit (IU) Serum. Dilute to contain 10, 40, and 100 IU in 5% BSA.

(2) Measles, Varicella and Rubella Controls

Select a panel of sera that have previously been tested and determined to be high or low positive or negative.

Individual serum samples or pooled sera may be used. Add 10 μ L of a 10% sodium azide solution per ml of serum (final dilution of sodium azide is 1:1,000). Store the sera at 4°C or –20°C. They are stable indefinitely unless they become contaminated.

Individual serum samples or sera to be pooled for controls should be screened for HB_s antigen and antibody to HIV before pooling. Positives should be eliminated or labeled accordingly.

Controls included on each plate:

MEASLES - standard, high-positive, low-positive, negative
RUBELLA - 10, 40, & 100 IU standard, negative, low-positive
VARICELLA - standard, high-positive, low-positive, negative

H. SPECIAL EQUIPMENT AND SUPPLIES

(1) Required equipment

- (a) Microplate reader, automated dual wavelength (405 nm/630 nm) (Biotek Model EL312E, Bio-Tek Instruments, Inc., Winooski, VT)
- (b) Computer for specimen entry, and to record and analyze data from plate reader. (IBM-Compatible with a minimum 486 CPU)
- (c) 96-well microplate washer with plastic carboys (2), tubing and traps. (Bio-Tek Model EL403, Bio-Tek Instruments, Inc., Winooski, VT)
- (d) Non-humidified incubator, 37°C \pm 1°C. (VWR Model 1520, VWR Scientific, San Francisco, CA)
- (e) Fan, household type, to dry 96-well microplates. (Patton HV-18C, Ace Hardware, Berkeley, CA)
- (f) Electronic digital pipette capable of dispensing 1 μ L to 250 μ L and tips (Rainin EDP-Plus EP-25 and EP-250, Rainin Instrument Co., Inc., Emeryville, CA).
- (g) Digital multi-channel pipette with ranges of 50–200 μ L and 5–50 μ L and racked tips (Labsystems Finnpipette Digital 12-Channel AS-8043 and Finntip 300 μ L tips AS-9341, Applied Scientific, South San Francisco, CA).

- (h) Microplates, 96-well, polystyrene, flat bottom, high binding capacity (Nunc strip well plates, NUNC #4-69949 or AS#72110, Applied Scientific, South San Francisco, CA).
- (i) Plastic microplate sealers (ICN/Flow Cat. #76-401-05, ICN/Flow Biochemicals, Inc., Costa Mesa, CA)
- (j) 96-well tube racks compatible with microplate format and 1-mL polypropylene microplate dilution tubes compatible with multichannel pipettor tips. (ICN/Flow Cat. #61-225-00, ICN/Flow Biochemicals, Inc., Costa Mesa, CA)
- (k) Plastic disposable multichannel pipette reagent reservoirs. (Costar #4870, Costar, Van Nuys, CA)
- (l) Pouch sealer, for polyester bags (Kapak 11214-108, VWR Scientific, San Francisco, CA).
- (m) Polyester, heavy duty, heat sealable, 8 x 10 or 6-1/2 x 8 inch bags (Kapak 11214-491 and 11214-527, VWR Scientific, San Francisco, CA).
- (n) Humidity indicating cards. (Multiform Dessicants, Inc., Buffalo, NY)
- (o) Silica gel desiccator packs, 5 gm. (Minipax dessicants #02-00040AG15, Multiform Dessicants, Inc., Buffalo, NY)
- (p) Plastic-backed paper cut for use as blotting pads. (KayDry 52857-120, VWR Scientific, San Francisco, CA).

(2) Other supplies

- (a) Glassware: 1000-, 500-, 200-, 100-mL bottles for storing solutions; 1000-, 400-, 250-, 100- and 50-mL beakers; 1000-, 500-, 250-, 100-, and 50-mL graduated cylinders; and 1000-, 500-, 250-, 100-, and 50-mL Ehrlenmeyer flasks.
- (b) Disposables: plastic weighing boats (various sizes); pipette tips, 1-250 μ L and 50-300 μ L capacities for Rainin and multichannel pipettes; pH standard buffers, 4.0, 7.0, 9.0; disposable glass or plastic pipettes, 10-, 5-, 2-, and 1-mL; Sarstedt 2-mL capped storage vials; kimwipes; and latex disposable gloves (various sizes and manufacturers).
- (c) Standard laboratory equipment and tools: magnetic stirrer; assorted size stirring bars; 4°C refrigerator; -70°C freezer; -20°C freezer; balances; pH meter and electrode; vortexes (Single tube and multi-tube); test tube racks; and 60 minute adjustable timer.

6. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration Curve

Not applicable for instrument operation.

B. Calibration Verification Procedure

(1) Spectrophotometer

Preventive maintenance is performed on the spectrophotometer (Bio-Tek EL312E) quarterly. The following tasks are performed by the manufacturer's representative according to manufacturer's specifications: optical verification; verification of alignment; repeatability test; accuracy test; and linearity test.

The manufacturer claims that "experience with this and similar instruments indicates that inaccuracies introduced by the environment or by laboratory techniques are far greater than any inaccuracies inherent in the basic instrument."

(2) Microplate washer

Preventive maintenance is performed on the microplate washer (Bio-Tek EL403) bimonthly. The following tasks are performed by the laboratory personnel according to manufacturer's specifications: Flushing with water and bleach to remove protein buildup; Decontamination of inflow/outflow tubing; Decontamination of washer and reservoirs; Adjustment of pressure delivery system, gravity feed system, and plate alignment to ensure proper washing.

7. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

(1) Import "Shipment Manifest Report" into MS ACCESS database.

(a) Prepare runsheets (weekly)

(b) Pull specimens for run (weekly)

(i) Remove pertinent boxes from freezer.

(ii) Remove specimens from boxes and check against map, then place specimens in blocks according to runsheet maps.

(iii) Place "repeat specimen" from earlier run in appropriate location in current block.

B. Sample preparation

(1) Add 597 μ L of serum diluent to 1-mL microplate tubes in 96-well rack for each patient specimen and control to be run.

(2) Add 3 μ L of each patient serum or control to tubes containing 597 μ L of diluent. (This is a 1:200 dilution of the specimen).

(3) Vortex all tubes.

(4) Dilutions may be made the day before the test and stored overnight at 4°C.

(5) Warm dilutions to room temp in 24°C water bath for 10 min prior to use.

C. Operation

(1) Prepare the plate washer according to the manufacturer's instructions.

(2) Remove the required number of appropriate (rubella or varicella-zoster coated) 96-well microplates and serum dilutions from the 4°C refrigerator and allow them to reach room temp before beginning test.

(3) Wash plates 3 times prior to use to remove excess antigen coating.

(4) Follow the runsheet map. Using a multichannel pipette, transfer 100 μ L of each diluted control serum and patient's serum to the appropriate wells coated with viral antigen and control antigen.

(5) Cover the plates with plastic sealers and incubate for 60 min in a 37°C incubator. In order to maintain timing, stagger plates at 3 min intervals.

- (6) Prepare the working dilution of conjugate in the conjugate diluent buffer.
 - (7) After 1 h, wash each plate in the plate washer (5x).
 - (8) Using a multichannel pipette and reservoirs, add 100 μ L of the conjugate dilution to each well. Maintain 3 min separation.
 - (9) Cover the plates with plastic sealers and incubate for 60 min in a 37°C incubator.
 - (10) Prepare the substrate working solution.
 - (11) After 1 h, remove plates from the incubator, remove seals and wash as in step 6. Do not allow plates to dry. Washed plates may be held inverted until all plates have been washed.
 - (12) Add 100 μ L substrate to each well.
 - (13) Cover and incubate in the 37°C incubator for 30 min.
 - (14) After 30 min remove plates, remove seals and add 100 μ L of the stopping solution (TSP) to each well.
 - (15) Read the microplates on the microplate reader set on 405 nm/630 nm (reference) with no blank and capture data from the reader into the computer using LABTRONICS COLLECT Software.
 - (16) Clean the microplate washer according to the manufacturer's instructions.
 - (17) Discard microplate strips in proper discard pan for autoclaving and discarding, keeping plastic frames for future use.
- D. Calculations (performed automatically by the computer program)
- (1) For each control serum and test specimen calculate the absorbance of the antigen well minus the absorbance of the control well (AG-NS value) and the ratio of the antigen well to the control well (AG/NS).
 - (2) Calculation of index values (Measles, Rubella & Varicella)
 - (a) Cut-off calculation

MEASLES - The cut-off value is 0.1.

RUBELLA - The mean AG-NS value of the duplicate 10 IU standards is used as the cut-off value.

VARICELLA - The cut-off value is 0.1.
 - (b) Calculate the O.D. INDEX by dividing the AG-NS value by the cut-off value for each serum.
$$\text{O.D. INDEX} = \frac{\text{viral antigen ABS} - \text{control antigen ABS}}{\text{cut-off value}}$$
 - (3) Calculation of International Units (IU) (Rubella)
 - (a) Perform a regression analysis and calculate a standard curve using the duplicate AG-NS values of the 10, 40, and 100 IU standards and their squares.

- (b) Predict the IU value for each control average and test sample from the standard curve by calculation.

E. Interpretation of Results:

- (1) An O.D. INDEX of ≥ 1.0 indicates the presence of antibody.
- (2) An O.D. INDEX of < 1.0 indicates that antibody was not detected.
- (3) An International Unit value of 10 or greater is considered significant for rubella.

8. REPORTABLE RANGE OF RESULTS

MEASLES

- (1) Index results range -0 to 30

RUBELLA

- (2) Index results range -0 to 30
- (3) International Unit range -0 to > 200 IU

VARICELLA

- (1) Index results range -0 to 30

9. QUALITY CONTROL (QC) AND QUALITY ASSURANCE (QA) PROCEDURES

A. Evaluation of control results

- (1) Measles - Evaluate the control wells and AG-NS values for each control serum
- (2) Rubella - evaluate the control wells and AG-NS value for each control serum. Evaluate the R squared value and predicted IU values for controls.
- (3) Varicella - Evaluate the control wells and AG-NS values for each control serum

B. Quality assurance (QA) procedures

- (1) Control Logs and Levey-Jennings Plots
- (2) Prepare a log of control results for each run including date tested; run number; mean net absorbance for each standard and control; and a notation when an individual control or run is unsatisfactory.
- (3) Calculate the mean and standard deviation for each control based on the first 40 acceptable runs with a lot of antigen.
- (4) Prepare a Levey-Jennings plot for each control with the mean from step 2 until two and four standard deviations are indicated. Plot the controls in batches of 40 runs. Plot all runs. The plots are referenced to the Control Log by log number.

C. Repeat Testing

- (1) Select at random one serum from each run for repeat testing. (2.56%)
- (2) Repeat measles, rubella, and varicella tests in the following day's runs.

10. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

A. Out-of-range results on control sera

- (1) When all control AG - NS values fall within the 2 SDs and the R squared and IU values are within limits, accept the run and report patient results.
- (2) If one or more of the controls exceeds the 2 SD limits, inspect control data and inspect patient results. It may be necessary to recalculate the results or reject the run and not report patient results. See supervising microbiologist.
- (3) If two or more control observations fall outside 4 SDs, reject the run and do not report results. Repeat run.

11. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

A. Non-specific reactions on patient's sera

- (1) Any reaction of the patient's sera with the control (NS) antigen >0.200 O.D. units is suspect. Inspect results for Ag/NS ratio to determine if results can be released.
- (2) High non-specific reactions should be individually evaluated to determine if they are influencing interpretation of results. If they are, the test is unsatisfactory. The serum should be absorbed and retested by EIA and by IFA.
- (3) Samples with AG-NS ratio <2.1 should be individually evaluated for non-specific reactions.

12. REFERENCE RANGES (NORMAL VALUES)

Not applicable.

13. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable.

14. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens reach and maintain ambient temp during testing. After analysis, the specimens are stored at -70°C .

15. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If the analytic system fails refrigerate the specimens at $4-8^{\circ}\text{C}$. until the analytical system is restored. If long-term interruption is anticipated, refreeze at -70°C .

16. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

A. Test Result Reporting:

- (1) All Test Results:

Data is compiled in MS ACCESS system and sent by e-mail attachment to the National Center for Health Statistics, or their designate, on a regular basis, within three weeks of receipt of specimens, as "shipments" are completed.

(2) Critical Calls

Not applicable.

17. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard record keeping means (e.g., electronic, mainframe, data files, laboratory notebook, floppy diskettes) are used to track specimens. It is recommended that records be kept for 8 years including QA/QC data and duplicate. The samples are stored at -70°C for 1 year, after analysis. They are packaged and mailed to the NCHS serum repository (McKesson Bioservices) in Rockville, MD. Only numerical identifiers are used; all personal identifiers are kept masked and available only to the project coordinator for safeguard confidentiality.

18. SUMMARY STATISTICS AND QC GRAPHS

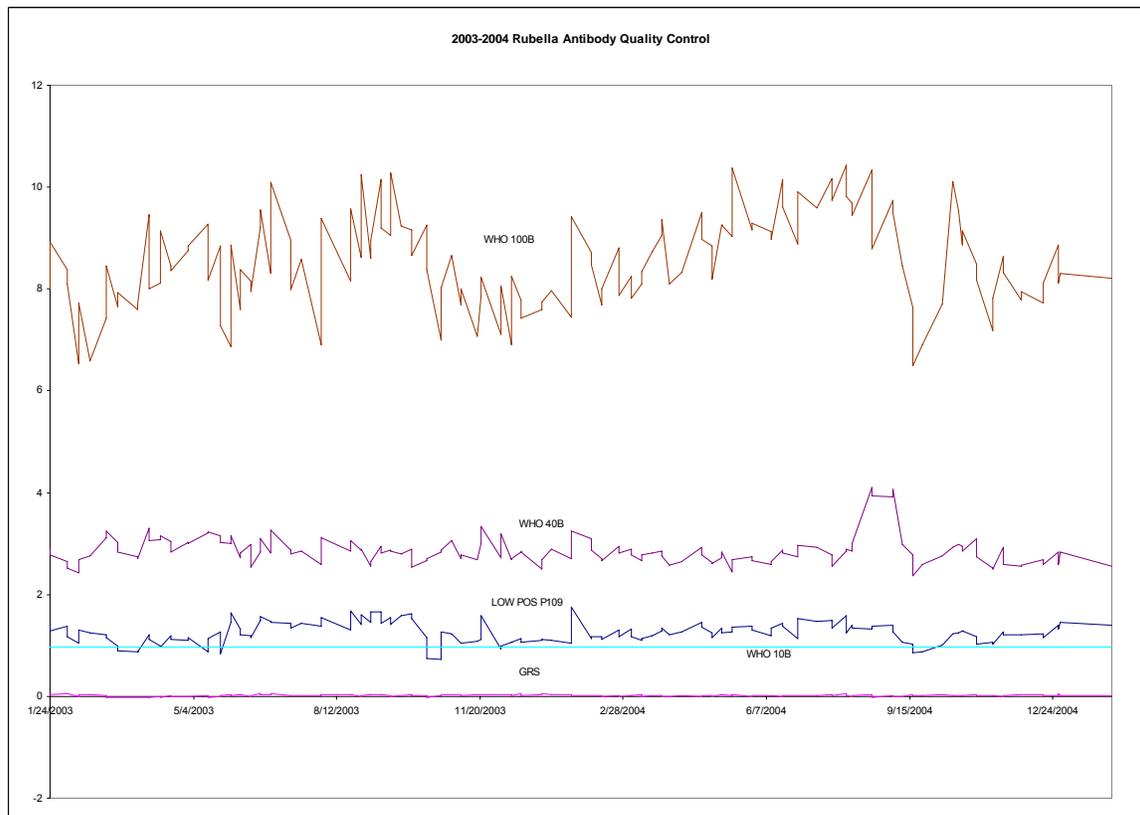
A. Measles

| Summary Statistics for Measles Antibody by Lot | | | | | | |
|--|-----|------------|----------|--------|--------------------|--------------------------|
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
| HU-152 | 139 | 1/24/2003 | 2/3/2005 | 0.073 | 0.028 | 38.4 |
| STD 1:5 | 139 | 1/24/2003 | 2/3/2005 | 3.289 | 0.38 | 11.5 |
| HU-PX | 139 | 1/24/2003 | 2/3/2005 | 3.394 | 0.323 | 9.5 |
| HU-153 | 139 | 1/24/2003 | 2/3/2005 | 13.132 | 1.258 | 9.6 |



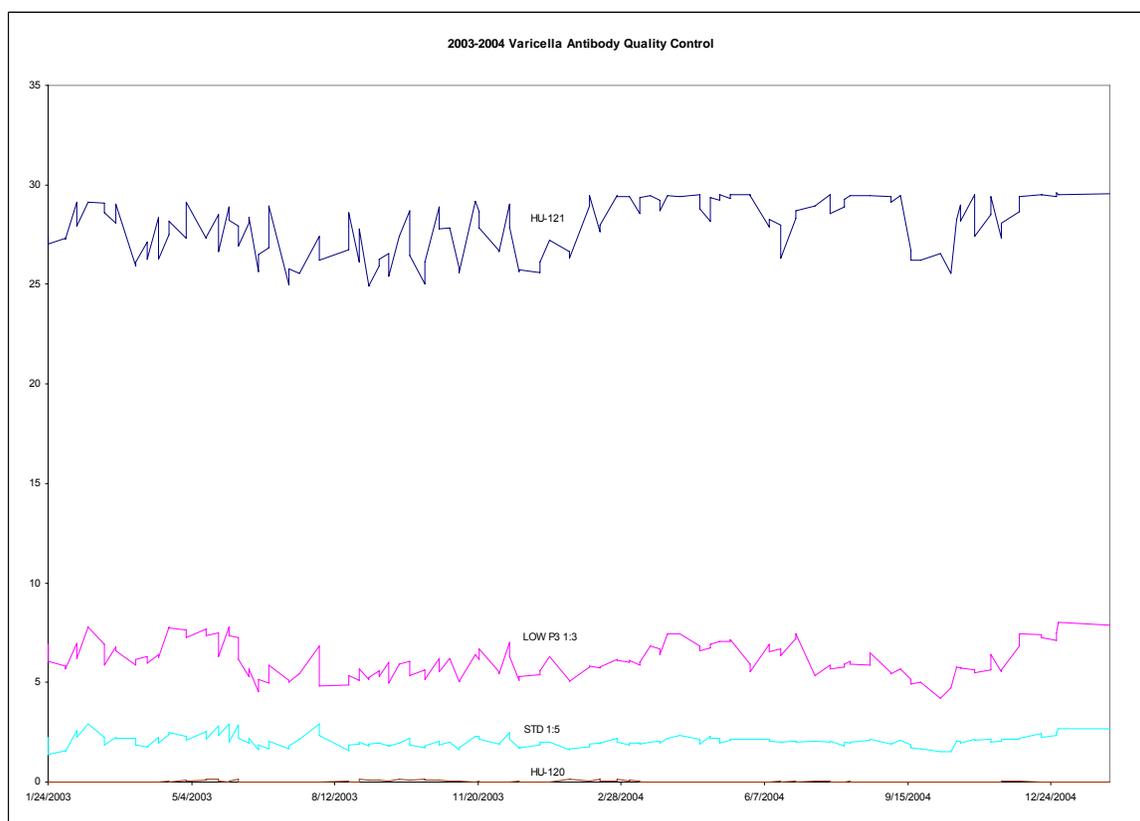
B. Rubella

| Summary Statistics for Rubella Antibody by Lot | | | | | | |
|--|-----|------------|----------|-------|--------------------|--------------------------|
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
| GRS | 139 | 1/24/2003 | 2/3/2005 | 0.016 | 0.02 | 123.8 |
| WHO 10B | 139 | 1/24/2003 | 2/3/2005 | 0.96 | 0 | 0 |
| LOW POS P109 | 139 | 1/24/2003 | 2/3/2005 | 1.249 | 0.204 | 16.3 |
| WHO 40B | 139 | 1/24/2003 | 2/3/2005 | 2.859 | 0.28 | 9.8 |
| WHO 100B | 139 | 1/24/2003 | 2/3/2005 | 8.535 | 0.896 | 10.5 |



C. Varicella

| Summary Statistics for Varicella Antibody by Lot | | | | | | |
|--|-----|------------|----------|--------|--------------------|--------------------------|
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
| HU-120 | 139 | 1/24/2003 | 2/3/2005 | 0.028 | 0.042 | 147.1 |
| STD 1:5 | 139 | 1/24/2003 | 2/3/2005 | 2.05 | 0.288 | 14.1 |
| LOW P3 1:3 | 139 | 1/24/2003 | 2/3/2005 | 6.137 | 0.847 | 13.8 |
| HU-121 | 139 | 1/24/2003 | 2/3/2005 | 27.906 | 1.378 | 4.9 |



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