SAM 405.05
Page 1 of 39

United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 405

Supplemental Assay Method for Detecting Lymphoid Leukosis Biocontamination by the COFAL Test

Date: August, 25, 2023

Number: SAM 405.05

Supersedes: SAM 405.04,November 28, 2014

Standard Requirement: 9 CFR, part 113.31

Contact: Email: Methodsrequest.notification@usda.gov
Phone: Center for Veterinary Biologics, 515-337-6100

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Supplemental Assay Method for Detecting Lymphoid Leukosis Biocontamination by the COFAL Test

Table of Contents

1. Introduction

2. Preparation for the Test

- 2.1 Personnel qualifications/training
- 2.2 Preparation of equipment/instrumentation

3. Part I. Propagation of Extraneous Lymphoid Leukosis Virus in Chick Embryo Fibroblasts (CEFs)

- 3.1 Equipment/instrumentation
- 3.2 Supplies
- 3.3 Reagents
- 3.4 Primary CEF preparation
- 3.5 Secondary CEF preparation
- 3.6 Preparation and inoculation of test material
  - 3.6.1 Avian encephalomyelitis (AE) vaccine (liquid glycerinated product)
  - 3.6.2 AE vaccine, lyophilized
  - 3.6.3 Hemorrhagic enteritis virus and marble spleen disease vaccine
  - 3.6.4 Infectious bronchitis vaccine, lyophilized or frozen
  - 3.6.5 Infectious bursal disease (IBD) vaccine, lyophilized or frozen
  - 3.6.6 Laryngotracheitis vaccine, lyophilized
  - 3.6.7 Marek's disease (MD) vaccine, cell associated, frozen
  - 3.6.8 MD vaccine, lyophilized
  - 3.6.9 Newcastle disease vaccine and combination Newcastle-bronchitis vaccine, lyophilized or frozen
  - 3.6.10 Pox and AE-pox combination vaccine
  - 3.6.11 Reovirus or tenosynovitis virus vaccine, lyophilized or frozen
  - 3.6.12 Recombinant virus vaccine
- 3.7 First and second harvest
- 3.8 Final harvest

4. Part II. Detection of Extraneous Lymphoid Leukosis Virus Group Specific (gs) Antigen by a Microtiter Complement Fixation (CF) Test

- 4.1 Equipment/instrumentation
- 4.2 Reagents/supplies

4.3 Preparation and standardization of reagents

4.3.1 Preparation of Veronal Buffered Diluent (VBD)
4.3.2 Preparation and standardization of 2.8% percent sheep red blood cell suspension
4.3.3 Preparation of hemoglobin color standards
4.3.4 Hemolysin titration
4.3.5 Preparation of sensitized cells
4.3.6 Complement (C') titration
4.3.7 Serum and antigen titrations

4.4 Detection of gs antigen in test material

5. Report of Test Results

6. Repeat Tests

7. References

8. Summary of Revisions

Appendices

I. Materials
II. Counting cells with a hemacytometer
III. Formulating cell suspensions

Supplemental Assay Method for Detecting Lymphoid Leukosis Biocontamination by the COFAL Test

1. Introduction

This Supplemental Assay Method (SAM) describes a procedure for the detection of lymphoid leukosis virus biocontamination in live virus poultry vaccines. The Complement Fixation for Avian Leukosis (COFAL) test is a 2-part procedure: Part I. Propagation of lymphoid leukosis virus, if present, in Chick Embryo Fibroblast (CEF) cell culture; and Part II. Detection of the group-specific (gs) antigen by a microtiter complement-fixation technique.

2. Preparation for the Test

2.1 Personnel qualifications/training

The personnel must have experience or training in this protocol. This includes knowledge of safe and aseptic biological laboratory techniques and preparation, proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals. The personnel must also have training in the operation of the necessary laboratory equipment required for this assay.

2.2 Preparation of equipment/instrumentation

Operate and monitor all equipment/instrumentation according to manufacturers' instructions. Maintain aseptic conditions in a laminar flow biological safety cabinet, and use sterile instruments and wear sterile gloves when appropriate.

3. Part I. Propagation of Extraneous Lymphoid Leukosis Virus in CEFs

3.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

3.1.1 Laminar Flow Biological Safety Cabinet (NuAire Inc., Labgard)

3.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model 252)

3.1.3 Water-jacketed incubator with a humidified 5% CO_2 atmosphere and temperature set at 37°C (Forma Scientific, Model No. 3158)

3.1.4 Roller apparatus (to rotate roller bottles)

3.1.5 Centrifuge (Beckman J6-MI, JS-4.2 rotor)

3.1.6 Vacuum pump (Curtin Matheson Scientific, Inc.)

3.1.7 Bunsen burner (Hanau Engineering Co., Touch-O-Matic)

3.1.8 Scissors, sterile (Roboz RS-6800)

3.1.9 Curved tip forceps, sterile (V. Mueller SU 2315)

3.1.10 Magnetic stir bars (sterile) and a stir plate

3.1.11 Hemacytometer (American Optical)

3.2 Supplies

Equivalent supplies may be substituted for any brand name listed below. All supplies must be sterile.

3.2.1 Tissue culture dish, 150 x 10-mm (Falcon, Cat. No. 1058)

3.2.2 Tissue culture dish, 100 x 20-mm (Falcon, Cat. No. 3003)

3.2.3 Plastic funnel covered with 4 layers of fine gauze

3.2.4 Conical tube, polypropylene, 29 x 114-mm, 50-mL (Sarstedt, Cat. No. 62.547.205)

3.2.5 Centrifuge tubes, polypropylene, 250-mL (Corning, Cat. No. 25350)

3.2.6 Roller bottles, 2000-mL, 850-cm^2, with screw-caps (Becton Dickinson, Cat. No. 3007)

3.2.7 Trypan Blue

3.2.8 Snap-cap tubes, 12 x 75-mm (Falcon, Cat. No. 2058)

3.2.9 Cell lifters (Costar, Cat. No. 3008)

3.2.10 Filtering flask, 1-L, with hose and cannula

3.2.11 Serological pipettes

3.2.12 Erlenmeyer flasks

3.2.13 Syringes and needles

3.2.14 Membrane filters, 0.22 µ (Millipore, Cat. No. SLGA025OS)

3.2.15 Membrane filters, 0.45 µ (Millipore, Cat. No. SLHA0250S)

3.2.16 Membrane filters, 5.0 µ (Millipore, Cat. No. SLAP02550)

3.3 Reagents

Equivalent reagents may be substituted. All reagents must be sterile.

3.3.1 Two dozen 9- to 11-day-old Specific-Pathogen-Free (SPF) chick embryos from a genetic line of chickens (C/O) which are susceptible to all lymphoid leukosis virus subgroups

3.3.2 Growth Medium

Medium 199 with Earles salts	1 L
Bacto tryptose phosphate broth	50 mL
$NaHCO_3$	1.5 g
Penicillin (potassium G)	100,000 units
Streptomycin sulfate	100 mg
Fetal bovine serum (FBS) (inactivated)	30-55 mL
Fungizone (optional)	2 mg

Adjust pH to 7.3 with $NaHCO_3$ solution

Note: Better cell growth and maintenance may occur if L-glutamine (10 mL/1000 mL growth medium = 1% L-glutamine) is added within 3 days prior to use of the medium.

If growth medium is used to wash embryonic tissues prior to trypsinization, omit the FBS and L-glutamine.

3.3.3 Puck's Saline A

NaCl	8.0 g
KCl	0.4 g
Glucose	1.0 g
Phenol Red (0.5% solution)	1.0 mL
q.s. with distilled or deionized water (DW)	1 L

Adjust pH to 7.2 with $NaHCO_3$ solution.

3.3.4 Ethylenediamine tetraacetate (EDTA) stock solution (1.0%), also referred to as versene

Add 1.0 g ethylenedinitrilo tetraacetic acid disodium salt to 100 mL Puck's Saline A.

3.3.5 Trypsin solution (0.25%)

Add 2.5 g trypsin (1:250) to 1 L Puck's Saline A.
Add 0.35 g $NaHCO_3$ per L.
Adjust pH to 7.4 with $NaHCO_3$ solution.

Note: For trypsinizing embryos, use 0.25% trypsin solution as is.

For trypsinizing primary cultures, add 2.0 mL of 1% EDTA to 100 mL 0.25% trypsin solution, or 0.25% trypsin may be used as is.

For trypsinizing secondary or higher passage chick embryo cell cultures, mix 0.25% trypsin 1:5 (1 part trypsin plus 4 parts Puck's Saline A) in Puck's Saline A. Then add 2.0 mL of 1.0%. EDTA solution per 100 mL trypsin solution. This makes a solution containing 0.05% trypsin and 0.02% EDTA. Add a few drops $NaHCO_3$ solution to bring the pH up to 7.2-7.4.

3.3.6 70% ethanol

3.4 Primary CEF preparation

Primary CEF cultures can be prepared from 9- to 11-day-old genetically susceptible (C/O) embryos from a COFAL-negative chicken flock in the following manner, or alternate methods of preparation are acceptable:

3.4.1 Disinfect the air cell end of the egg with 70% ethanol, flame, and break open the shell with sterile forceps. Use the forceps to open the membranes, lift out the embryo, and place it in a sterile disposable 150 x 10-mm tissue culture or petri dish. Four to 24 embryos may be prepared together. Remove (and discard) the heads and viscera of the embryos with sterile scissors and forceps. Wash the embryos several times with a 0.25% trypsin solution (room temp), a physiological saline solution, or growth medium without FBS or L-glutamine. Place the washed embryos in a sterile dry 150 x 10-mm tissue culture or petri dish and mince them thoroughly using sharp sterile scissors.

3.4.2 To further wash, put the minced tissue into a trypsinizing flask containing 50 mL growth medium without FBS or L glutamine and a magnetic stir bar. Place

the flask on a stir plate and stir with a moderate vortex for 5 minutes. Allow the cells to settle and decant (and discard) the supernatant and repeat.

3.4.3 To trypsinize the tissues, first rinse the residual media from the cells by adding 25 mL of the 0.25% trypsin solution, and then immediately decant the trypsin solution. Next, add 50 mL of the 0.25% trypsin solution to the flask and mix on a magnetic stir plate for 20 minutes. (Set stir plate to produce a moderate vortex.)

3.4.4 Place a sterile gauze wrapped funnel into the opening of a 250-mL conical centrifuge tube. To stop the trypsinizing action on the cells, pour 2 mL of FBS through the gauze and then pour the trypsinized contents of the flask through the gauze funnel into the centrifuge tube. Bring the total volume to approximately 125 mL with growth media. Centrifuge for 10 minutes at 250 x *g* with the temperature set at 10°C.

3.4.5 Observe and record the volume of packed cells, and then pour off the supernatant. Dilute the cells approximately 1:300 with growth medium. Plant 200 mL of diluted cell suspension in each 1000-mL roller bottle. Tighten the cap and incubate for 4 days in an incubator set at 37°C. (Alternately, if tissue culture flasks are planted, adjust the volume of cell suspension appropriately, leave the caps loose, and incubate in a humidified incubator with the temperature set at 37°C and the atmosphere set at 5% CO_2 for 4 days.) After 4 days, the cell sheet should be well proliferated and ready to split.

3.5 Secondary CEF preparation

Secondary CEF cultures can be prepared in the following manner, or alternate methods of preparation are acceptable:

3.5.1 Decant the medium from 1 roller bottle of primary CEFs, pipetting any remaining media with a 5-mL pipette, and add 15 mL of the 0.25% trypsin solution prewarmed to 37°C. Rotate the bottle until the cells begin to detach, approximately 1 minute. (The proper length of time will be learned by experience. Too short a time will result in large clumps of cells in the new suspension.) Decant the trypsin and, with an open hand, strike the side of the roller bottle until most of the cells become detached. Rinse the inside of the roller bottle with 15 mL of growth medium and swirl. Pipette the suspension with the cells into an empty Erlenmeyer flask. Repeat twice more for a total of 3 rinses. Repeat the above steps separately for each additional roller bottle.

3.5.2 Pour the combined cell suspension from the Erlenmeyer flask through a funnel covered with 4 layers of fine gauze into a second Erlenmeyer flask containing a sterile stir bar and 25 mL of growth medium for each roller bottle used. Then rinse the funnel with an additional 25 mL of medium for each roller bottle used. Thoroughly mix the cell suspension.

3.5.3 Make a cell count with a hemocytometer. (A total cell count using crystal violet stain is satisfactory.) See the **Appendices.** Adjust the volume so that the cell concentration is approximately 500,000 cells per mL.

3.5.4 Plant the secondary cell suspension into the vessels to be used for the test (usually 60-mm or 100-mm plastic tissue culture dishes). Use 5 mL of the suspension for a 60-mm dish and 15 mL of the suspension for a 100-mm dish. The cultures are now ready to be inoculated with test materials.

3.6 Preparation and inoculation of test material

This protocol is designed primarily to detect infective lymphoid leukosis virus contamination in live virus poultry vaccines. Preparation procedures described are those which are least complicated, but still have been found in most cases to be adequate for the purpose. These procedures are tentative and will of necessity be changed as more knowledge and experience dictates. A suggested procedure for each type of vaccine is described separately. If the test materials are to be diluted for inoculation, regular growth medium or medium without serum can be used. In each case, add the inoculum to the plates in addition to the growth medium already in the plates. The test material may be inoculated immediately after planting the cell cultures if experience has shown the material used will not be toxic to chick embryo fibroblasts. After inoculation, incubate the cultures in a humidified incubator set at 37°C and containing 5% CO_2. Sixteen to 24 hours later, remove the fluids and replace with fresh growth medium added at the original planting volume of 5 mL per 60-mm plate or 15 mL per 100-mm plate.

Note: With materials that may be somewhat toxic, allow the cell sheets to attach and grow for 18 hours prior to adding the test inoculum, and then leave inoculum on the cells for 4 to 24 hours depending on the toxic effect.

3.6.1 Avian encephalomyelitis (AE) vaccine (liquid glycerinated product)

This product tends to be somewhat toxic to the cell cultures, and the procedure for inoculation needs to be adjusted accordingly. Dilute the vaccine with tissue culture medium so that 1000 doses are present in 20 mL total volume. Centrifuge the mixture for 30 minutes at 1800 x *g*.

The solids will be primarily in the bottom, the fat- containing portion on top, and an aqueous portion in the middle. Insert a pipette into the aqueous portion and remove more than 10 mL. Filter this through a 5.0-micron membrane filter.

Divide a quantity of filtrate equal to 500 doses (10 mL) among 5, 60-mm plates or 2, 100-mm plates. With this product, it is not known how much, if any, the vaccine virus or other vaccine components interfere with infection of the cells by leukosis virus.

3.6.2 AE vaccine, lyophilized

Restore this product with 1.0 mL tissue culture medium per 100 doses vaccine. If the product contains coarse tissue elements, centrifuge the restored material for 20 minutes at 1000 x *g* and filter through a 0.45-micron membrane filter. Divide an amount of inoculum equal to 500 doses among 5, 60-mm plates or 2, 100-mm plates. To date, none of the products of this type have adversely affected the cell cultures. How much, if any, interference to infection of the cells by leukosis virus is caused by this vaccine virus is not known.

3.6.3 Hemorrhagic enteritis virus and marble spleen disease vaccine

Thaw or restore and dilute these products with 1.0 mL tissue culture medium per 100 doses vaccine. If the product contains coarse tissue elements, centrifuge the restored material for 20 minutes at 1000 x *g* and filter through a 0.45 micron membrane filter. Divide an amount of inoculum equal to 500 doses among 5, 60-mm plates or 2, 100-mm plates. To date, none of the products of this type have adversely affected the cell cultures. How much, if any, interference to infection of the cells by leukosis virus is caused by these vaccine viruses is not known.

3.6.4 Infectious bronchitis vaccine, lyophilized or frozen

Thaw or restore and dilute these products with 1.0 mL tissue culture medium per 100 doses vaccine. If the product contains coarse tissue elements, centrifuge the restored material for 20 minutes at 1000 x *g* and filter through a 0.45-micron membrane filter. Divide an amount of inoculum equal to 500 doses among 5, 60-mm plates or 2, 100-mm plates. To date, none of the products of this type have adversely affected the cell cultures. How much, if any, interference to infection of the cells by leukosis virus is caused by this vaccine virus is not known.

3.6.5 Infectious bursal disease (IBD) vaccine, lyophilized or frozen

Thaw or restore and dilute this product with 1.0 mL tissue culture medium per 100 doses vaccine. Centrifuge the restored material for 20 minutes at 1000 x *g* and filter through a 0.45-micron membrane filter. Neutralize the IBD virus with high titer specific antiserum (antiserum must be free of leukosis-sarcoma viruses and their antibodies). Mix 2 mL of the restored vaccine (200 doses) with 3 mL of the antiserum and incubate at room temperature for 1 hour. In some instances, a higher proportion of antiserum may be necessary. After the incubation period, inoculate the mixture, equivalent to 200 doses, into the cell cultures, dividing the entire amount equally among 5, 60-mm plates or 2, 100-mm plates.

3.6.6 Laryngotracheitis vaccine, lyophilized

Restore this product with 1.0 mL tissue culture medium per 100 doses vaccine. Centrifuge the restored material for 20 minutes at 1000 x *g*, and then filter through a 0.22-micron membrane filter. Divide an amount of inoculum equal to 500 doses among 5, 60-mm plates or 2, 100-mm plates.

3.6.7 Marek's disease (MD) vaccine, cell associated, frozen

Thaw and dilute vaccine to 100 doses per 1.0 mL in tissue culture medium. Centrifuge 10.0 mL of the inoculum for 20 minutes at 1000 x *g* and filter through a 0.22-micron membrane filter. Incubate at 25°- 30°C for 20 to 30 minutes. Divide a quantity of filtrate equal to 500 doses among 5, 60-mm plates or 2, 100-mm plates.

3.6.8 MD vaccine, lyophilized

Restore this product with 1.0 mL tissue culture medium per 100 doses vaccine. Centrifuge the restored material for 20 minutes at 1000 x *g* and filter through a 0.22-micron membrane filter. Neutralize 500 doses of the Marek's disease vaccine virus with high titer specific antiserum (antiserum must be free of leukosis-sarcoma viruses and their antibodies) and incubate at room temperature for 1 hour. After the incubation period, inoculate the mixture into the cell cultures, dividing the entire amount equally among 5, 60-mm plates or 2, 100-mm plates.

3.6.9 Newcastle disease vaccine and combination Newcastle-bronchitis vaccine, lyophilized or frozen

Thaw or restore and dilute these products with 1.0 mL tissue culture medium per 100 doses vaccine. Neutralize the Newcastle disease virus (NDV) with high titer specific antiserum (antiserum must be free of leukosis-sarcoma viruses and their antibodies). Mix 2 mL of the restored vaccine (200 doses) with 2 mL of the NDV antiserum and incubate at room temperature for 1 hour. If the Newcastle vaccine is of particularly high titer, a higher proportion of antiserum may be necessary. After the incubation period, inoculate the mixture into the cell cultures, dividing the entire amount equally among 5, 60-mm plates or 2, 100-mm plates.

3.6.10 Pox and AE-pox combination vaccine

An optimum method for effectively neutralizing, inactivating, or separating out pox virus has not been determined, but the following method may be attempted. Thaw or restore and dilute these products with 10.0 mL tissue culture medium per 500 doses vaccine. Centrifuge for 20 minutes at 1000 x *g* and filter through a 0.22-micron membrane filter. Divide an amount of inoculum equal to 500 doses among 5, 60-mm plates or 2, 100-mm plates.

3.6.11 Reovirus or tenosynovitis virus vaccine, lyophilized or frozen

An optimum method for effectively neutralizing, inactivating, or separating out this virus has not been determined, but the following method may be attempted. Thaw or restore and dilute these products with 1.0 mL tissue culture medium per 100 doses vaccine. Centrifuge the restored material for 20 minutes at 1000 x *g* and filter through a 0.45-micron membrane filter. Neutralize the reovirus or tenosynovitis virus with high titer specific antiserum (antiserum must be free of leukosis-sarcoma viruses and their antibodies). Mix 2 mL of the restored vaccine (200 doses) with 2 mL of the antiserum and incubate at room temperature for 1 hour. In some instances, a higher proportion of antiserum may be necessary. After the incubation period, inoculate the mixture, equivalent to 200 doses, into the cell cultures, dividing the entire amount equally among 5, 60-mm plates or 2, 100-mm plates).

3.6.12 Recombinant virus vaccine

Follow the same method as for the vectoring agent.

Note: With each test series, maintain both positive and negative controls. Positive controls consist of 2 sets of plates, 1 inoculated with a subgroup A lymphoid leukosis virus, and 1 inoculated with a subgroup B lymphoid leukosis virus. Maintain a set of uninoculated plates as a negative control.

In all cases, remove the inoculum and medium in 24 hours or less after inoculating test material and add fresh growth medium.

Allow the cultures to grow for another 4 or 5 days without further treatment unless the pH of the medium becomes acid, necessitating a medium change.

At the end of this time, the first harvest of test material is made and the remaining cells are subcultured.

3.7 First and second harvest

3.7.1 Using an inverted microscope**,** examine each dish for contamination by bacteria or molds and to observe the condition of the cell sheet. Discard all contaminated plates and those with poor cellular growth. Harvest samples and passage cells as per **Sections 3.7.2-3.7.3**. Alternate methods are acceptable provided appropriate samples are harvested and cells are split and transferred. It is not acceptable to seed the split cells onto a new cell sheet of CEFs.

Note: Process only 1 set of plates at a time and in the following order: Negative controls, test samples, and then positive controls.

3.7.2 Tip the culture dishes slightly and, using a suction apparatus, remove all but approximately 0.25 mL of culture fluid from a 60-mm dish or 0.75 mL from a 100-mm dish. Use a sterile cell lifter and scrape off 1/2 of the cell sheet from each plate into the remaining fluids. Remove and pool these cell suspensions for each set of test plates. Freeze these pools at -60°C or colder until the complement fixation (CF) test is conducted.

3.7.3 Subculture the remaining cells in the following manner: Add 0.05% trypsin (trypsin/Puck's Saline, 1:5) plus 0.02% versene solution (3 mL per 60-mm dish, 5 mL per 100-mm dish) to each dish. Allow the trypsin solution to remain in contact with the cell sheet for 1 to 2 minutes, swirling occasionally, and then remove it immediately when the cells begin to detach from the plate. Let the dishes stand at room temperature until the cells loosen further. (Strike the plates sharply with the palm of the hand to facilitate cell detachment.) Add fresh growth medium to the dishes, and disperse the cells with gentle pipetting. Pool these cell suspensions, mix thoroughly, and plant into new dishes. Plant 1 new culture dish for each original culture dish (a 2-for-1 split as 1/2 the cells were saved for testing). Incubate these cultures at 37°C as before.

Note: The cultures will usually grow for 1 week without a medium change, but if the pH becomes too acid, the medium will have to be changed in the interim. At the end of this week period, handle the cultures exactly the same way as at the first subculture. Should the growth rate be so rapid that the cell sheet begins to peel before the scheduled time for subculture, an additional harvest and subculture may be made.

3.8 Final harvest

After this second subculture (postinoculation), maintain the cultures under the same conditions as before until the 21st day post inoculation, at which time a final harvest is conducted. At this final harvest, leave twice the fluid in each plate as before and scrape off the entire sheet with a cell lifter. Divide the final harvest (each test) and store a portion at -60°C or lower until after CF testing of the other portion is completed. If the CF testing of any particular series is inconclusive, the stored portion of the final harvest may be used as an inoculum for a new test series.

Cell culture fluids from each subculture or medium change are tested for extraneous lymphoid leukosis by the CF test.

4. Part II: Detection of Extraneous Lymphoid Leukosis Virus Group Specific (gs) Antigen by a Microtiter Complement Fixation (CF) Test

The CF test method described has been adapted from the Laboratory Branch Complement Fixation (LBCF) Method developed at the National Communicable Disease Center, Atlanta, Georgia. The LBCF method is described in detail in Public Health Monograph No. 74. A supplement to this is entitled, "A Guide to the Performance of the Standardized Diagnostic Complement Fixation Method and Adaptation to Micro Test." First edition, July 1, 1969, has been prepared by the same Laboratory Section. These documents may be available from Public Health Inquiries Branch, U. S. Public Health Service, Washington, D.C. 20201.

4.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

4.1.1 Centrifuge (Beckman J6-MI, JS-3.0 rotor)

4.1.2 Incubator CO_2 (Forma Scientific, Model No. 3158)

4.1.3 Freezer, -70°C (Revco Scientific, Model No. ULT1790-7-ABA)

4.1.4 Water bath, 37°± 1°C

4.1.5 Mini-Orbital shaker (Bellco Glass, Cat. No. 7744-S0010)

4.1.6 Vortex mixer (Thermolyne Maxi Mix II, Model No. M37615)

4.1.7 Reading mirror (Cooke Engineering Co. Microtiter)

4.1.8 12-channel pipette (Labsystems Model Finnpipette Digital Multichannel 50-300 µL)

4.1.9 Pipettes (Labsystems Model Finnpipette Digital 5-40 µL & 40-200 µL)

4.1.10 Plate cover roller

4.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

4.2.1 Guinea pig complement (Whittaker Bioproducts, Cat. No. 30-956J)

4.2.2 Anti-sheep hemolysin and diluent (Baltimore Biological Laboratory)

4.2.3 Sheep blood (25 mL Alsevers + 25 mL sheep blood) 4 to 31 days after collection

4.2.4 DW

4.2.5 Graduated Kolmer centrifuge tube, 10-mL (Corning Cat. No. 8360 or Kimbal Cat. No. 45180)

4.2.6 Graduated cylinder, 100-mL

4.2.7 Erlenmeyer flasks, 125- and 250-mL

4.2.8 Beaker, 50-mL

4.2.9 Tissue culture 96-well U-shaped plate (Linbro, Cat. No. 76-013-05)

4.2.10 Adhesive plate cover (Dynatech Laboratories, Cat. No. 001-010-3501)

4.2.11 Polypropylene conical tube, 50-mL, 29 x 114-mL, sterile, (Sarstedt, Cat. No. 62.547.205)

4.2.12 Disposable polystyrene centrifuge tubes, 15-mL, 17 x 100-mL tubes (Falcon, Cat. No. 2057)

4.2.13 Disposable borosilicate serologic tubes, 3-mL, 12 x 75-mm

4.2.14 Snap-cap tubes, 3-mL, 12 x 75-mm

4.2.15 Disposable serological pipettes, 1-, 5-, 10- and 25-mL

4.2.16 Pipette tips

4.2.17 Log-log paper

4.2.18 Plain graph paper

4.3 Preparation and standardization of reagents

4.3.1 Preparation of Veronal Buffered Diluent (VBD)

1. Preparation of the stock buffer solution (5X VBD)

a. Combine the following in a 2-L volumetric flask in the order listed:

NaCl	83.00 g
Na-5, 5-diethyl barbiturate	10.19 g
DW	1500 mL
1 N hydrochloric acid	34.58 mL
Stock solution containing 1.0 molar $MgC1_2$ and 0.3 molar $CaC1_2$ (20.3 gm $MgCl_2 \cdot 6H_2O$ and 4.4g $CaC1_2 \cdot 2H_2O$ in 100 mL DW)	5.00 mL

b. Fill to the 2-L mark with DW. Mix thoroughly.

c. Check the pH of the stock buffer before refrigeration by making a 1:5 dilution with DW (1 part 5X VBD plus 4 parts distilled water). The pH of the diluted stock (1X VBD) must be 7.3-7.4. If the pH is not in this range, discard and prepare fresh stock buffer.

2. Preparation of gelatin-water solution

a. Add 1.0 g of gelatin to 100 mL of DW. Bring to a boil to dissolve the gelatin.

b. Make up to 800 mL with sterile DW at room temperature.

c. Chill in the refrigerator. This solution should not be held longer than 1 week.

3. Preparation of VBD (lX) for daily use (containing 0.1% gelatin)

Add 4 volumes of gelatin water to 1 volume of 5X VBD stock buffer. Store in the refrigerator. VBD (1X) should not be stored longer than 24 hours.

Note: The pH of the VBD (1X) must be 7.3 to 7.4.

4.3.2 Preparation and standardization of 2.8% sheep red blood cell suspension

1. Washing sheep cells (collected and preserved in modified Alsever's solution, aged 4 days)

a. Add 2 or 3 volumes of cold VBD (1X) to each volume of preserved cells and centrifuge at 600 X *g* for 5 minutes.

b. Carefully aspirate the supernatant fluid and the white cell layer without disturbing the erythrocytes.

c. Fill centrifuge tube again with cold VBD. Thoroughly resuspend the cells by gently mixing with a pipette. Centrifuge at 600 X *g* for 5 minutes and repeat the process for a total of 3 washings. If the supernate is not colorless after the second washing, cells are too fragile and must not be used.

d. Resuspend the cells once more in cold VBD and centrifuge for 10 minutes at 600 X g to pack the cells.

e. Record the volume of packed cells in the centrifuge tube, and aspirate the supernate. Care should be taken to remove as much fluid as possible without disturbing the cells.

2. Standardization of 2.8% cell suspension

a. Prepare the 2.8% cell suspension (approximately 670,000 cells/mm^3) by adding 34.7 volume of VBD to 1 volume of packed sheep cells (**Illustration 1**). Shake flask gently to insure even suspension of cells.

Supplemental Assay Method for Detecting Lymphoid Leukosis Biocontamination by the COFAL Test

ILLUSTRATION 1

Standardization of Sheep Red Blood Cells to 2.8%

Cell Pack Volume (mL)	Adjust to Total Volume with VBD (mL)
0.5	17.9
0.6	21.4
0.7	25.0
0.8	28.6
0.9	32.1
1.0	35.7
1.1	39.3
1.2	42.8
1.3	46.4
1.4	50.0
1.5	53.6
1.6	57.1
1.7	60.7
1.8	64.3
1.9	67.8
2.0	71.4
2.1	75.0
2.2	78.5
2.3	82.1
2.4	85.7
2.5	89.3
2.6	92.8
2.7	96.4
2.8	100.0
2.9	103.5
3.0	107.1
3.1	110.7
3.2	114.2
3.3	117.8
3.4	121.4
3.5	125.0
3.6	128.5
3.7	132.1
3.8	135.6
3.9	139.2
4.0	142.8

b. To check the density of the 2.8% cell suspension, pipette 7.0 mL into a 10-mL graduated Kolmer centrifuge tube (either a Corning 8360 or Kimbal 45180 has acceptable tolerance) and centrifuge at 600 X *g* for 10 minutes. A 7.0 mL aliquot of properly prepared cell suspension should produce 0.2 mL of packed cells.

c. When the volume of packed cells is under or above the 0.2 mL point, the cell suspension should be adjusted. The quantity of VBD which must be added to or removed from the cell suspension is determined by the following formula, where PCV = actual packed cell volume of 7.0 mL

(PCV/0.20 mL) x 100 mL = corrected volume

Example 1, low density: PCV = 0.19 mL

$$\frac{0.19 \text{ mL}}{0.20 \text{ mL}} \times 100 \text{ mL} = 95.0 \text{ mL}$$

Therefore, remove 5 mL of VBD from each 100 mL of cell suspension. (VBD may be removed by centrifuging an aliquot of the cell suspension and pipetting off the desired amount for discard.)

Example 2, high density: PCV = 0.21 mL

$$\frac{0.21 \text{ mL}}{0.20 \text{ mL}} \times 100 \text{ mL} = 105.0 \text{ mL}$$

Therefore, add 5.0 mL of VBD to each 100 mL of cell suspension.

Check the adjusted cell suspension by centrifuging a 7.0 mL portion as before.

Note: Keep the cell suspension in the refrigerator when not in use. Always swirl the flask gently before use to secure an even suspension of the erythrocytes.

4.3.3 Preparation of hemoglobin color standards

Eleven color standards are used to read the hemolysin, complement, and antigen titrations. These standards are prepared by combining proportionate amounts (**Illustration 2**) of hemoglobin solution and 0.28% cell suspension as follows:

1. **Preparation of hemoglobin solution**

   a. Pipette 1.0 mL of the 2.8% cell suspension into a large test tube (17 x 100-mm).

   b. Add 7.0 mL DW and shake the mixture until all cells are lysed.

c. Add 2.0 mL of the stock buffer solution (5X VBD) to restore tonicity and mix thoroughly.

2. Preparation of 0.28% cell suspension

Pipette 1.0 mL of the 2.8% cell suspension into a large test tube, add 9.0 mL of VBD, and mix thoroughly.

3. Preparation of color standards

a. Label 11 serologic tubes (12 x 75-mm) with the specified % of hemolysis shown in **Illustration 2**. Mix the varying ratios of hemoglobin solution and the 0.28% cell suspension as shown.

ILLUSTRATION 2

Reagents	Percent hemolysis										
	0	10	20	30	40	50	60	70	80	90	100
Hemoglobin solution	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
0.28 % cells	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0

b. Shake the tubes and centrifuge at 600 X *g* for 5 minutes. Remove from the centrifuge without agitation. When not in use, store in refrigerator to prevent excessive change in color. The color standards, prepared for reading the complement titration, may be used the next day to read tests.

4.3.4 Hemolysin titration

1. Preparation of 1:100 hemolysin (rabbit antiserum against sheep red cells) solution

Thoroughly mix 98.0 mL of VBD and 2.0 mL of glycerinated hemolysin (lyophilized hemolysin with its glycerine containing diluent is equivalent). Dispense in convenient small aliquots and store at -20°C or lower. Do not refreeze after thawing.

2. Determination of hemolysin dilution needed for sensitization of 2.8% sheep cells

Perform a hemolysin titration each time a new lot of 1:100 hemolysin solution is prepared and each time a new lot of sheep cells is used.

a. Place 6 serologic tubes (12 x 75-mm) in a rack and label with hemolysin dilutions shown in last column of **Illustration 3**. Prepare dilutions for titration as shown. Thoroughly mix each dilution.

Note: These final hemolysin dilutions have been found appropriate for commercially prepared hemolysins. If they are not satisfactory, prepare other dilutions that suffice for the particular lot of hemolysin in routine use.

ILLUSTRATION 3

Preparation of Hemolysin Dilutions

Hemolysin dilution, 1 mL	Diluent, mL	Final hemolysin dilution
1:100---------plus	9.0--------gives	1:1000
1:1000-------plus	1.0--------gives	1:2000
1:1000-------plus	1.5--------gives	1:2500
1:1000-------plus	2.0--------gives	1:3000
1:1000-------plus	3.0--------gives	1:4000
1:1000-------plus	7.0--------gives	1:8000

b. Label a second set of 6 serologic tubes with the correct hemolysin dilutions. Add 1.0 mL of the standardized 2.8% sheep cell suspension to each tube.

c. Add 1.0 mL of each of the final hemolysin dilutions (1:1000 through 1:8000) to the 1.0 mL portions of sheep cell suspension with constant swirling of the contents. Incubate for 15 minutes at 37ºC. After the incubation period, the sheep erythrocytes are “sensitized cells," ready for use.

d. Make a 1:400 dilution of guinea pig complement (C') by drawing up undiluted C' in a 1.0-mL pipette beyond the 0.6 mL mark. Wipe the tip of the pipette and return excess C' above the 0.6 mL mark to the stock container. Deliver 0.25 mL of C', without using the last 0.1 mL of graduation of the pipette, into 99.75 mL of cold VBD. The diluted C' must be kept cold and be used within 2 hours.

Note: With less active C', it may be necessary to use a 1:300 dilution, or with a more potent C', a 1:500 dilution may be needed for proper results.

e. Label 6 serologic tubes with the final hemolysin dilutions. To each tube add 0.4 mL of cold VBD, 0.4 mL of the 1:400 C' dilution, and 0.2 mL of cells sensitized with the series of final hemolysin dilutions (**Step c**) to the properly labeled tube. Mix and incubate in a 37°C water bath for 30 minutes. Centrifuge at 600 X *g* to pack the cells. Read the % hemolysis in each tube by comparing with the color standards.

f. Plot the amount of hemolysis obtained with each dilution of hemolysin on ordinary graph paper. Determine the optimal dilution of hemolysin from the graph by inspection. It is desirable to select a dilution such that further increase in hemolysin (as the curve progresses to the right) does not appreciably change the % lysis. The dilution selected also needs to provide for a slight excess of hemolysin. See **Illustration 4**.

Note: On the X-axis, let the left end of the hemolysin dilution scale be 0, and measure a suitable length for the 1:1000 dilution. Other dilutions are represented as fractions of this length. Thus 1:2000 = 1/2 the length of 1:1000; 1:2500 = 2/5 of 1:1000; 1:3000 = 1/3 of 1:1000; 1:4000 = 1/4 of 1:1000; and 1:8000 = 1/8 of 1:1000.

ILLUSTRATION 4

Hemolysin titration

Note: Increasing the hemolysin concentration (left to right) with complement held constant results in a "plateau" from which the optimal hemolysin dilution is selected. The optimal hemolysin dilution in this instance is 1:2000.

4.3.5 Preparation of sensitized cells

1. Add 1 volume of optimal hemolysin dilution (example, 1:2000 dilution shown in **Illustration 4**) to 1 volume of the standardized 2.8% cell suspension with rapid, but gentle, swirling.

2. Before using, incubate for 15 minutes at 37°C.

4.3.6 Complement (C') titration

Note: For routine qualitative testing, the following procedure for handling complement has been found to be satisfactory: Commercially obtained lyophilized guinea pig complement is restored as directed. A 1:20 dilution of restored C' is made in 1X VBD; this dilution is dispensed in convenient (amount to be used at one time for tests) aliquots into inert plastic vials and

stored at -70°C or colder. The C' dilution is thawed (10°C water bath) and further diluted for titration. After the titer is determined, the remaining vials are thawed and diluted as needed for test use. A vial of each batch so prepared should be titered every 2 weeks to check for changes. There may be no significant decrease in titer over a period of several months.

Titration of C' is performed as follows:

1. Prepare sensitized cells.

2. Label 4 serologic (12 x 75-mm) tubes as in **Illustration 5**. To obtain accuracy, titrate in duplicate and use the average hemolysis.

3. Prepare 1:400 dilution of C'. (The 1:400 dilution of C' prepared previously and stored in the refrigerator may be used for titrations of complement and hemolysin when performed at the same time.)

4. Add reagents in the order shown in **Illustration 5**.

5. Shake tubes and place in the 37°C water bath for 30 minutes. Shake once at 15 minutes.

ILLUSTRATION 5

Complement Titration

Reagent	Tube Number			
	1	2	3	4
VBD	0.6 mL	0.55 mL	0.5 mL	0.4 mL
1:400 dilution of C’	0.2 mL	0.25 mL	0.3 mL	0.4 mL
Sensitized cells	0.2 mL	0.2 mL	0.2 mL	0.2 mL

6. Remove tubes from water bath and centrifuge 600 X *g* to pack the cells. Determine the % hemolysis in each tube by comparing with the color standards, interpolating to the nearest 5% when a tube does not exactly match 1 of the standards.

ILLUSTRATION 6

Conversion of % lysed cells, y, to ratio of % lysed to non-lysed cells, y/100-y

y	y/100-y	y	y/100-y	y	y/100-y
10	0.111	40	0.67	70	2.33
15	0.176	45	0.82	75	3.00
20	0.25	50	1.0	80	4.0
25	0.33	55	1.22	85	5.7
30	0.43	60	1.50	90	9.0
35	0.54	65	1.86		

7. Record the % hemolysis (y) for each tube. Using the chart in **Illustration 6**, determine the ratio of % lysed to non-lysed cells, y/100-y, for each volume (mL) of the 1:400 C', as shown in the example below. The "y" values are hypothetical.

Example:

Tube #	Vol (mL)of 1:400 C'	% Hemolysis (y)	Ratio (y/100-y)
1	0.20	25	0.33
2	0.25	40	0.67
3	0.30	70	2.33
4	0.40	85	5.70

8. For each of the 4 tubes, plot on log-log graph paper the volume of the 1:400 dilution of C' in mL against the ratio found in the chart. (Do not graph when "y" exceeds 90% nor when "y" is less than 10%.) Join the 2 points plotted for the first 2 tubes and find the midpoint as shown in **Illustration 7**. Do the same for the last 2 tubes. (A good titration should have 2 points on each side of 1). Draw a line through the 2 midpoints. Draw a horizontal line through the intersection of this line with the vertical "I" line and read the $C'H_{50}$ in mL of the 1:400 dilution of C'. Five $C'H_{50}$ are required in the diagnostic test.

In the example, $C'H_{50}$ is 0.27 mL. Five units are contained in 1.35 mL (5 X 0.27) of the 1:400 dilution. The dilution of C' necessary to obtain 5 $C'H_{50}$ in 0.4 mL is calculated as follows:

400 = X **1.35 X = 160**

X = 118.5 or 119

1.35 0.4

Hence 0.4 mL of a 1:119 dilution of C' contains 5 $C'H_{50}$. (Direct readings for proper dilutions may be found in **Illustration 8**.)

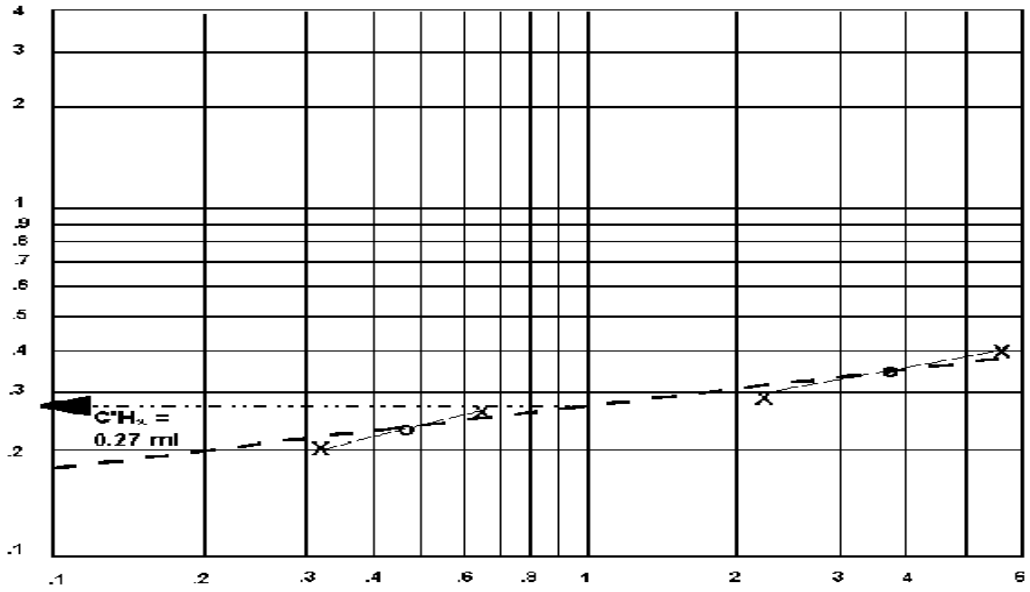
Note: 0.4 mL of this working dilution of C' contains 5 units of $C'H_{50}$ when reacted with 0.2 mL sensitized 2.8% sheep red blood cells, as titrated above. 0.05 mL (50 µl) of this working dilution results in the same concentration (5 units of $C'H_{50}$) when reacted with 0.025 mL (25 µl) sensitized 2.8% sheep red blood cells, as used in the COFAL microtiter assay (Sections 4.3.7 and 4.4).

9. Determine the slope of the line joining the midpoints of the C' titration plot. First, measure from any point near the left end of the line 10 cm horizontally to the right. Then measure the vertical distance upward from the right end of the horizontal line to the slope line. Divide the vertical measurement by 10 cm to obtain the slope (1/N). The normal value for a valid slope is 0.20 ± 10%.

Supplemental Assay Method for Detecting Lymphoid Leukosis Biocontamination by the COFAL Test

ILLUSTRATION 7

Graphing Method for Complement Titration



[use log-log paper]

y axis = mL volume of 1:400 dilution of C'
x axis = the ratio "%Lysed / %non-lysed cells" (y/100-y)

ILLUSTRATION 8

Complement Dilutions for Conversion of 1 C'H_{50} to 5 C'H_{50s} in the LBCF Test

ML C' - 1 C'H_{50}	For 5 C'H_{50s}, Dilute Stock C'				
	1:300 C'	1:350 C'	1:400 C'	1:450 C'	1:500 C'
0.21	114	133	152	171	190
0.22	109	127	145	164	182
0.23	104	122	139	157	174
0.24	100	117	133	150	167
0.25	96	112	128	144	160
0.26	92	108	123	138	153
0.27	89	104	119	133	148
0.28	86	100	114	129	143
0.29	83	97	110	124	139
0.30	80	93	107	120	133
0.31	77	90	103	116	129
0.32	75	87	100	113	125
0.33	73	85	97	109	121
0.34	71	82	94	106	118
0.35	69	80	91	103	114

4.3.7 Serum and antigen titrations

The serum is a standard antiserum for the test, and the antigen is a known gs antigen-containing reagent. To conserve serum, the titrations are done in a microtiter block test system.

1. **Preparation of serum for inactivation**

   a. Make an initial dilution (in 1X VBD) of positive serum which will be fourfold lower (4X) than the stated titer (if known or estimated).

   b. Inactivate the diluted serum for 30 minutes at 56°C.

   c. The diluted and inactivated antiserum may be dispensed into convenient small aliquots (sufficient for a day's test) and stored at -70°C or lower.

2. Addition of VBD to plates (see **Illustration 9**)

a. Add 25 µL (0.025 mL) VBD to the wells in which the antigen dilutions are to be made, except the first one in each row. (In **Illustration 9** this includes wells 2 to 8, A through H). Also add 25 µL to the 3 wells of the serum-complement controls (9A through 9C) and to the 5 wells of serum control (9D through 9H).

b. Add 50 µL (0.05 mL) VBD with a pipette dropper to each of the 3 wells of the VBD complement controls (10A through 10C)

c. Add 100 µL (0.1 mL) VBD with a pipette dropper to a well for the cell control (10H).

ILLUSTRATION 9 - Template for serum and antigen titrations

Anticomplementary Controls		1	2	3	4	5	6	7	8	9	10	11	12
1.25 $C'H_{50}$	A									Serum (4X) C' Control	VBD Control	X	0%
2.5 $C'H_{50}$	B									Serum (4X) C' Control	VBD Control	X	10%
5.0 $C'H_{50}$	C									Serum (4X) C' Control	VBD Control	X	20%
Serum dilutions $\frac{X}{4}$	D									X/4 serum control	X	X	30%
$\frac{X}{2}$	E									X/2 serum control	X	X	40%
X	F									X serum control	X	100%	50%
2X	G									2X serum control	X	90%	60%
4X	H									4X serum control	Red cell control	80%	70%
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256			Hemolysis Standards	

Antigen Dilutions

3. Addition of antigen to plates

a. Add 25 µL of the test antigen to the first 2 wells of each row (1 and 2, A through H, **Illustration 9**).

b. Using a pipetter set at 25 µL (or a 0.025 microdiluter), mix and make serial twofold dilutions starting with wells 2A through 2H and continuing to wells 8A through 8H. Discard the 25 µL after mixing the number 8 wells.

4. Addition of serum to plates

a. Make serial twofold dilutions in tubes of the serum prepared in Step 1. These dilutions will be 4X (the concentration as prepared in Step 1), 2X, X (the expected titration endpoint), X, and X.2 4

b. Add 25 µL of the corresponding dilution of serum to the wells as shown in **Illustration 9**. The dilutions will be added to wells 1 through 9 in the appropriate row (rows D-H). The 4X concentration will also be added to wells 9A through 9C.

c. Shake the plates and allow to stand at 4°C until the complement is added. Stack or cover plates to prevent evaporation.

5. Preparation and addition of complement

a. Make the dilution of complement which will contain 5 $C'H_{50}$ in 0.4 mL (see **Section 4.3.6**). From this, make a 1:2 and a 1:4 dilution to give 2.5 and 1.25 units, respectively.

b. Add 50 µL of this complement dilution to all wells getting 5 units of complement (wells 1-9, rows C through H in **Illustration 9**).

c. Add 50 µL of a 1:2 dilution of complement to all wells getting 2.5 units of C' (wells 1-9, row B).

d. Add 50 µL of a 1:4 dilution of complement to all wells getting 1.25 units of C' (wells 1-9, row A).

e. Shake the plates and incubate at 4°- 6°C for 15 to 18 hours. Stack or seal plates to avoid evaporation.

6. Addition of sensitized red cells

a. Add 25 µL of sensitized sheep red blood cells to all the wells. Shake immediately, tape, and incubate at 37°C.

Note: Incubation may be done in a water jacketed incubator, or by floating the plates in a 37°C water bath for 30 minutes. The plates should be shaken gently every 10 minutes to keep the cells (remaining) in suspension or the plates may be placed on a mechanical shaker in a 37°C incubator for the 30 minutes period. A shaker with a reciprocal action traversing a course of 2-3 mm at approximately 250-500 times per minute will keep the cells in suspension and not cause splashing. More vigorous shaking must be avoided to prevent mechanical lysis of cells or inactivation of complement.

b. After incubation, add 125 µL (0.125 mL) of each color standard to the plates.

c. Centrifuge plates at 600 X *g* for 5 minutes or allow cells to settle at 4°C for 4 to 6 hours.

d. Read by comparison with color standards. Record % hemolysis on record sheets and determine optimal dilution of antigen and titer of the serum. Use 4 units of antiserum for each test.

4.4 Detection of gs antigen in test material

Store all test samples, including positive and negative controls, at -60°C or colder until the CF test is conducted. Just prior to testing, thaw and refreeze each sample (test sample and control samples) 3 times to disrupt intact cells and to release group specific antigen, if present.

The antiserum used in these tests is one produced in rabbits against the P-27 antigen of an avian leukosis virus. This reagent will be supplied by the Center for Veterinary Biologics (CVB).

4.4.1 Test wells

Add reagents in the following order: (See **Illustration 10**)

1. Add 25 µL VBD to all test wells, rows A-H, wells 1-9.

2. Using a pipetter set at 25 µL (or a 0.025-mL microdiluter), add appropriate antigen suspension to the first well of each test series and make the 1:2, 1:4, 1:8 dilutions. Change the pipette tips (or blot the diluter) and pick up another aliquot of antigen to make the 1:2 anti-complementary test dilution.

3. Add 25 µL of 4X or 4 unit antiserum to all test wells receiving antiserum (not the anti-complementary test wells). Add 25 µL for VBD to the anti-complementary test wells.

4. Shake plates and stack to prevent evaporation until complement is added.

5. Prepare working dilution of C' as determined in **Section 4.3.6**. Add 50 µL to all the test wells (rows A-H, wells 1-9). This is best done late in the afternoon. Shake plates and incubate at 4°C for 15 to 18 hours. Stack or cover to prevent evaporation.

6. Add 25 µL of the prepared presensitized red cells to all wells. Tape and seal plates. Place on a slow speed reciprocal shaker at 37°C and agitate constantly (with just enough force to keep the cells from settling) for 30 minutes. Remove from incubation and centrifuge at 600 X *g* for 5 minutes.

7. Read by comparison with color standards and record % hemolysis.

4.4.2 Control wells

Only 1 set of controls is needed for each group of tests. These additions are made at the same time as the test wells.

1. In Row 12, add VBD to the following: 25 µL to wells A and B', 50 µL to wells C, D, and E', and 100 µL to well F.

2. Add 25 µL antiserum (4 units) to wells 12A and 12G.

3. Add 25 µL of a positive antigen control (4 units) to wells 12B and 12G.

4. Add 50 µL working dilution complement to wells 12A, 12B, 12C, and 12G. Dilute complement 1:2 and add 50 µL to well 12D. Dilute complement 1:4 and add 50 µL to well 12E.

5. Add 25 µL sensitized red cells to all wells and proceed as in the screen- ing tests. For samples of results of completed test see **Illustration 11**.

ILLUSTRATION 10

Template for CF plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1:2									X	0%	Serum A/C
B	1:4									X	10%	Pos. Ag A/C
C	1:8									X	20%	5 $C'H_{50}$
D	A/C 1:2									X	30%	2.5 $C'H_{50}$
E	1:2									X	40%	1.25 $C'H_{50}$
F	1:4									100%	50%	Red cell control
G	1:8									90%	60%	Pos. Ag Pos. Serum
H	A/C 1:2									80%	70%	X

Color Stds

Supplemental Assay Method for Detecting Lymphoid Leukosis Biocontaminationby the COFAL Test

ILLUSTRATION 11 - Sample plate readings

	N-1 1	N-2 2	N-3 3	S1-1 4	S1-2 5	S1-3 6	S2-1 7	S2-2 8	S2-3 9	10	11	12	
A 1:2	100	100	100	0	0	0	100	100	100	X	0%	100	Serum A/C
B 1:4	100	100	100	30	0	0	100	100	100	X	10%	100	Pos. Ag. A/C
C 1:8	100	100	100	70	10	0	100	100	100	X	20%	100	5 C'H_{50}
D A/C 1:2	100	100	100	100	100	100	100	100	100	X	30%	100	2.5 C'H_{50}
E 1:2	100	30	20	0	0	0	0	0	0	X	40%	50- 70	1.25 C'H_{50}
F 1:4	100	80	40	0	0	0	0	0	0	100%	50%	0	Red cell control
G 1:8	100	100	100	20	0	0	30	0	0	90%	60%	0	Pos. Ag. Pos. Serum
H A/C 1:2	100	100	100	100	100	100	100	100	100	80%	70%	X	
	S3-1	S3-2	S3-3	R1-1	R1-2	R1-3	R2-1	R2-2	R2-3	Color stds			

N = uninoculated culture S = test sample culture
R = positive control culture Number in square = % hemolysis

5. Report of Test Results

Presence of complement-fixing activity in the harvested samples (from passages) at the 1:4 or higher dilution, in the absence of anticomplementary activity, is considered positive unless the activity can definitely be established to be caused by something other than infectious lymphoid leukosis virus. Activity at the 1:2 dilution is considered to be suspicious, and the sample must be further subcultured to determine the presence or absence of the group-specific antigen.

5.1 Record the results as percentages for each dilution of each passage, and report the final results of each test as negative/satisfactory or positive/unsatisfactory.

5.2 Report "no test" in case of reagent or control failure and specify which.

6. Repeat Tests

The decision to repeat a test will be made according to the merits of each situation.

7. References

7.1 Sarma, P. S., H. C. Turner, and R. J. Huebner. An avian leukosis group specific complement-fixation reaction. Application for the detection and assay of non-cytopathogenic leucosis viruses. Virology 23:313-321 (1964).

7.2 Public Health Monograph No. 74. Standardized diagnostic complement fixation method and adaptation of micro test (1965). U.S. Department of Health, Education and Welfare, Washington, D.C. 20201.

7.3 A Guide to the Performance of the Standardized Diagnostic Complement Fixation Method and Adaptation to Micro Test. 1st Edition, July 1, 1969. U.S. Department of Health, Education, and Welfare, Washington, D.C. 20201.

7.4 Sever, J. L. Application of a microtechnique to viral serological investigations. J. Immunol. 88:320-329 (1962).

7.5 Title 9, *Code of Federal Regulations*, section 113.31, U.S. Government Printing office, Washington, DC.

8. Summary of Revisions

Version .05

- **The coversheet and contact information has been updated.**

Version .04

- The Contact information has been updated; however, the Virology Section has elected to keep the same next review date for the document.

Version .03

- The contact information was updated.

Version .02

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- The document number has been changed from PYSOP0405 to SAM 405.
- The Contact information has been updated.

Version .01

This document was completely revised and rewritten in new format to 1) meet the current NVSL/CVB QA requirements, 2) update the reagent and equipment lists, 3) allow for alternate methods of cell culture preparation and handling, 4) include treatment methods for additional vaccines, and 5) to provide additional testing clarification. No significant changes were made from the superseded protocol.

Appendices: Counting Cells in Suspension with a Neubauer Hemacytometer and Formulating Cell Suspensions

The protocol in this appendices describe a method to count cells in suspension with the use of a 1/10 mm deep Neubauer hemacytometer and microscope. It also explains how to formulate cell suspensions for specific concentrations and volumes.

Appendix I

Materials

1. Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

1. Hemacytometer (1/10 mm deep Neubauer hemacytometer)

2. Incandescent microscope (capable of 100X magnification

3. Hand Tally Counter (Daigger Cat. No. GX6594)

2. Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

1. Trypan Blue (0.4%)

2. Snap-cap tube, 6-mL, 12 x 75-mm, (Falcon, Cat. No. 2058)

3. Disposable plastic pipettes, 1-, 5-, 10-, and 25-mL

4. Pipette (Labsystems, Model Finnpipette Digital 5- to 40-µL)

5. Disposable pipette tips

Supplemental Assay Method for Detecting Lymphoid Leukosis Biocontamination by the COFAL Test

Appendix II

Counting cells with a hemacytometer

In a 6-mL snap-cap tube, dilute 0.5 mL of the cell suspension to be counted with 1.0 mL of 0.4% Trypan Blue solution. Replace the cap on the tube and mix the suspension by inverting the tube several times. Place the cover slip over the counting grid on the hemacytometer. While the cells are still suspended, quickly load the chamber of the hemacytometer with this preparation using a pipette. Fill the chamber with enough of the preparation to completely cover the grid under the cover slip. Overfilling can cause erroneous counts. The total volume of cell suspension in 10 1-mm squares (see numbered squares) under the cover slip is equal to 1 cubic mm or 1 µL.

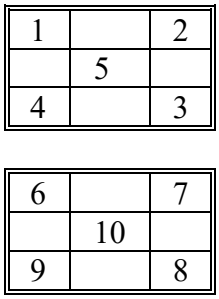


Figure 1.

Let the preparation stand for approximately 1 minute to allow the cells to settle onto the bottom of the counting chamber. Examine the grid with a microscope under 100X magnification. Check for the even distribution of cells. (Any irregularity of distribution will cause erroneous results.) Count the live cells (dead cells stain blue while live cells remain translucent). Count only those live cells that fall on the top line, the left line, or within each numbered 1-mm square (1-10) in the grid (**Figure 1**). Keep track of the total number of cells counted for the 10 squares with a Hand Tally Counter.

Calculate the number of cells per ml in the initial suspension by multiplying the total cell count by the dilution in Trypan Blue (3) and by the conversion factor from cubic mm µL to mL (1000). See example below.

Example:

number of cells in 10 squares= 385
1:3 cell dilution= x 3
1155
cubic mm/mL x 1000
number of cells per mL = 1155000

Appendix III

Formulating cell suspensions

1. First calculate the Dilution Factor (DF) by dividing the concentration of cells/mL in the initial suspension by the desired final concentration of cells/mL.

Example:

If the concentration of cells in the initial suspension is 1155000 cells/mL and the desired final concentration is 350000 cells/mL, then:

DF = 1155000 cells/mL divided by 350000 cells/mL = 3.3

2. Next, divide the desired final volume of cells by the DF. The result of this division will tell you the volume of the initial cell suspension you will need to make the final desired volume of cells.

Example:

If the final desired volume of cells is 375 mL, then:

375 mL/3.3 (DF) = 113.6 mL

This is the volume of the initial cell suspension needed to make the final volume of cells. (Round this number to 114.)

3. Finally, take the amount of the initial cell suspension determined in **Step 2** and add sufficient media to bring it to the final volume of cells desired.

Example:

Add 261 mL (375 mL - 114 mL) of media to 114 mL of the initial cell suspension (1155000 cells/mL) to get 375 mL of the desired 350000 cells/mL suspension.