

United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Detecting Extraneous Avian Leukosis Virus in Biologic Products by p27
ELISA

Date: October 2, 2014
Number: VIRPRO0415.03
Supersedes: VIRPRO0415.02, November 27, 2012
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Detecting Extraneous Avian Leukosis Virus in Biologic Products by p27 ELISA

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Detecting Extraneous Avian Leukosis Virus in Biologic Products

1. Introduction

This Testing Protocol (PRO) describes a procedure for the detection of extraneous avian leukosis virus (ALV) in biologic products. The following procedure is divided into two parts: Part I, propagation of potential extraneous ALV in cell culture; and Part II, detection of the group-specific antigen (p27) by enzyme-linked immunosorbent assay (ELISA).

2. Preparation for the Test

2.1 Personnel qualifications/training

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. 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 manufacturer's 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 Chick Embryo Fibroblasts (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

3.1.2 Humidified, rotating egg incubator

3.1.3 Water-jacketed incubator with a humidified 5% CO₂ atmosphere and temperature set at 37°C

3.1.4 Roller apparatus (to rotate roller bottles)

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

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- 3.1.6** Vacuum pump
- 3.1.7** Scissors, sterile
- 3.1.8** Curved tip forceps, sterile
- 3.1.9** Magnetic stir bars (sterile) and a stir plate
- 3.1.10** Hemacytometer

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
- 3.2.2** Tissue culture dish, 100 x 20-mm
- 3.2.3** Plastic funnel covered with 4 layers of fine gauze
- 3.2.4** Conical tube, polypropylene, 29 x 114-mm, 50-mL
- 3.2.5** Centrifuge tubes, polypropylene, 250-mL
- 3.2.6** Roller bottles, 2000-mL, 850-cm², with screw-caps
- 3.2.7** Trypan Blue
- 3.2.8** Snap-cap tubes, 12 x 75-mm
- 3.2.9** Cell lifters
- 3.2.10** Serological pipettes
- 3.2.11** Erlenmeyer flasks
- 3.2.12** Syringes and needles
- 3.2.13** Membrane filters, 0.22- μ
- 3.2.14** Membrane filters, 0.45- μ

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3.2.15 Disposable plastic pipettes 1-, 5-, 10-, and 25-mL

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 which are susceptible to all exogenous ALV 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 |
| Newborn calf serum or equivalent | 30-55 mL |
| Fungizone (optional) | 2 mg |

Adjust pH to 7.3 with NaHCO₃ 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 | 1 L |

Adjust pH to 7.2 with NaHCO₃ solution.

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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 NaHCO₃ per L.

Adjust pH to 7.4 with NaHCO₃ 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 NaHCO₃ 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/E) embryos from an ALV-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 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. Remove and discard the heads and viscera of the embryos with sterile forceps. Wash the embryo carcass several times with growth media (without NCS or L-glutamine) to remove excess blood. Place the washed embryos in a sterile dry 100 x 10-mm tissue culture or petri dish and mince them thoroughly using sharp sterile scissors.

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3.4.2 To further wash, put the minced tissue into a trypsinizing flask containing 50 mL growth medium (without NCS 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 10 mL of the 0.25% trypsin solution, and then immediately decant the trypsin solution. Next add 40 mL of the 0.25% trypsin solution to the flask and mix on a magnetic stir plate for 15 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 NCS 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 (1050 rpms using a Beckman J6-MI centrifuge with a JS-4.2 rotor) with the temperature set at 10°C.

3.4.5 Observe and record the volume of packed cells, and then remove the supernatant using a 25-mL pipette. 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% CO₂ 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.

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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 75 mL of growth medium for each roller bottle used. Then rinse the funnel with an additional 75 mL of medium for each roller bottle used. Thoroughly mix the cell suspension.

3.5.3 Quantitate the cell count of the suspension by following the procedure described in the **Appendix**. Adjust the volume so that the cell concentration is approximately 200,000 to 300,000 cells per mL.

3.5.4 Plant the secondary cell suspension into the vessels to be used for the test using 15 mL of the suspension for a 100-mm dish. Incubate the cultures in a humidified incubator set at 37°C and containing 5% CO₂.

3.6 Preparation and inoculation of test material

This protocol is designed primarily to detect extraneous exogenous ALV 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% CO₂. Sixteen to 24 hours later, remove the fluids and replace with fresh growth medium added at the original planting volume of 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. Antisera used to neutralize vaccine virus must be heat inactivated to inactivate cytotoxic complement.

3.6.1 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 (2400 rpms using a Beckman J6-MI centrifuge with a JS-4.2 rotor) and filter through a 0.45- μ membrane filter. Divide an amount of inoculum equal to 500 doses among 2 x 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.

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3.6.2 Hemorrhagic enteritis virus and marble spleen disease vaccine

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* (2400 rpms using a Beckman J6-MI centrifuge with a JS-4.2 rotor) and filter through a 0.45- μ membrane filter. Divide an amount of inoculum equal to 500 doses among 2 x 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.

3.6.3 Infectious bronchitis vaccine, lyophilized or frozen

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* (2400 rpms using a Beckman J6-MI centrifuge with a JS-4.2 rotor) and filter through a 0.45- μ membrane filter. Divide an amount of inoculum equal to 500 doses among 2 x 100-mm plates.

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

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* (2400 rpms using a Beckman J6-MI centrifuge with a JS-4.2 rotor) and filter through a 0.45- μ 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 2 x 100-mm plates.

3.6.5 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* (2400 rpms using a Beckman J6-MI centrifuge with a JS-4.2 rotor), and then filter through a 0.22- μ membrane filter. Divide an amount of inoculum equal to 500 doses among 2 x 100-mm plates.

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

Thaw and dilute vaccine 1.0 mL tissue culture medium per 100 doses vaccine. If it is a Master Seed Virus, dilute 1 to 1 with media. Freeze and thaw 3X. Centrifuge at 2400 rpm/ 20 min in Beckman J6-M1 centrifuge. Pipette off supernatant from pellet and discard pellet. Heat inactivate supernatant at 39°C ($\pm 2^\circ\text{C}$) in a water bath for 2 hours. Divide half the sample between 2 x 100 mm plates of CEFs. Change medium on plates at approximately 18 to 24 hours as per usual procedure.

Note: The freeze-thaw, centrifuge, and heat-inactivation steps are performed for each harvest.

3.6.7 MD vaccine, lyophilized

Restore this product with 1.0 mL tissue culture medium per 100 doses vaccine. Freeze and thaw 3X. Centrifuge at 2400 rpm/20 min in Beckman J6-M1 centrifuge. Pipette off supernatant from pellet and discard pellet. Heat inactivate supernatant at 39°C ($\pm 2^\circ\text{C}$) in a water bath for 2 hours. Divide half the sample between 2 x 100 mm plates of CEFs. Change medium on plates at approximately 18 to 24 hours as per usual procedure.

Note: The freeze-thaw, centrifuge, and heat-inactivation steps are performed for each harvest.

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

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 2 x 100-mm plates.

3.6.9 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. Dilute these products with 10.0 mL tissue culture medium per 500 doses vaccine. Centrifuge for 20 minutes at 1000 x g (2400 rpms using a Beckman J6-MI centrifuge with a JS-4.2 rotor) and filter through a 0.22- μ membrane filter. Divide an amount of inoculum equal to 500 doses among 2 x 100-mm plates.

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3.6.10 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 (2400 rpms using a Beckman J6-MI centrifuge with a JS-4.2 rotor) and filter through a 0.45- μ 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 2 x 100-mm plates.

3.6.11 Recombinant virus vaccine

Follow the same method as for the vectoring agent.

3.7 Controls and initial media change

3.7.1 With each test series, maintain both positive and negative controls. Positive controls consist of a minimum of 1 set, inoculated with either ALV subgroup A or ALV subgroup B. Other appropriate ALV controls maybe used in addition to ALV subgroups A and B. Also maintain a set of uninoculated plates as a negative control.

3.7.2 Within 24 hours after inoculating plates, remove and discard the culture medium from all plates. Replace the discarded culture medium with equivalent volume of fresh growth medium.

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

3.7.3 Incubate the cultures an additional 5 to 6 days without further treatment unless the medium becomes acidic requiring a medium change.

Note: It is highly recommended to microscopically observe the cultures several times after inoculation of the original material and during subsequent harvest and subcultures of cells.

3.7.4 After the additional incubation, harvest the first sample of culture material and subculture the remaining cells as per **Sections 3.8.2 and 3.8.3**

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3.8 First and second harvest

3.8.1 Microscopically examine each plate for extraneous microbial contamination and 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 3.8.2 and 3.8.3**.

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

3.8.2 Tip the culture dishes slightly and using a pipette, remove all but approximately 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 and store these samples at -60°C or colder until the detection of ALV virus group-specific antigen (p27) by ELISA is conducted as per **Section 4**.

3.8.3 Subculture the remaining cells in the following manner: Add 0.05% trypsin (trypsin/Puck's Saline, 1:5) plus 0.02% versene (EDTA) solution (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 covered 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. Use a total of 5 mL for the 100-mm dishes. Pool these cell suspensions, mix thoroughly, and plant into 2 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). Add 15 mL growth media to each 100-mm dish. Incubate these cultures at 37°C as before for 1 week.

Note: The cultures will usually grow for 1 week without a medium change, but if the pH becomes too acidic, the medium will have to be changed in the interim. At the end of this week period, repeat Sections 3.8.1 through 3.8.3. 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.

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3.9 Final harvest

A final harvest is performed at the 21st day postinoculation. At this final harvest, remove all but approximately 2-3 mL of fluid in each plate and scrape off the entire cell sheet with a cell lifter. Divide the final harvest (each test sample) and store a portion at -60°C or lower until after p27 ELISA testing of the other samples is completed. Cell culture fluids from each subculture are tested for extraneous ALV by the p27 ELISA as per **Section 4**. If the p27 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.

4. Part II: Detection of Extraneous ALV Virus Group-specific (gs) Antigen by p27 ELISA

The following procedure describes a commercial antigen capture enzyme-linked immunosorbent assay (ELISA) kit to test for the presence of extraneous avian leukosis virus (ALV) p27 antigen in biologic products.

4.1 Equipment/instrumentation

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

4.1.1 Commercial ALV p27 antigen ELISA test kit (Affinitech Ltd, ALV 1000, ALV 0500, ALV 0200); IDEXX ALV antigen test kit 99-09254; Synbiotics Corporation, 96-6524) or equivalent.

4.1.2 12-channel pipette, 850- μ L

4.1.3 Pipette tips

4.1.4 Plate reader

4.2 Reagents/supplies

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

4.2.1 Distilled or deionized water

4.2.2 Pipette tips

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4.3 Detection of group specific p27 antigen in test material using ELISA

4.3.1 All test samples, including positive and negative controls, should be stored at -60°C or colder until the p27 ELISA test is conducted. Just prior to testing, thaw and refreeze each sample (test samples and control samples) **3 times** to disrupt intact cells and release group specific p27 antigen, if present.

4.3.2 Follow the instructions provided with the commercial ALV ELISA test kit to test samples and controls. If there is more than one wash method listed in the instructions, use the one with the most washes. Using a microplate reader, read and record the absorbance of the wells at the wavelength listed in the kit instructions.

4.3.3 The kit positive and negative control mean absorbances are determined. See the kit instructions for the optimal absorbance values for the negative and positive controls to determine if the test is valid.

4.3.4 Sections 4.3.5 through 4.3.6 describe additional validity requirements for the test to accommodate the off-label testing of cell culture lysates as they are not listed as a sample on the test kit inserts.

4.3.5 The mean absorbance readings from each set of cell negative control wells **for each cell passage** are calculated. For the test to be valid, the difference between the mean absorbance of the **kit positive control** minus the mean **cell culture negative control** for each passage should meet or exceed the mean absorbance listed in the kit instructions.

4.3.6 To determine the relative antigen level in the samples (including the controls), the sample to positive (S/P) ratio needs to be calculated. The following formula compensates for possible “background” in the cell culture lysate test samples and controls. The calculation is as follows:

$$S/P = \frac{(\text{Sample Mean} - \text{Cell Culture Negative Control Mean})}{(\text{Kit Positive Control Mean} - \text{Cell Culture Negative Control Mean})}$$

Note: For each cell culture passage, the negative control mean subtracted must be from the same passage level or harvest as the sample mean used for calculating the S/P ratio. For example, subtract 1st passage cell culture negative mean from 1st passage sample mean, subtract 2nd passage cell culture negative mean from 2nd passage sample mean, etc.

4.3.7 ALV cell culture positive controls must have an S/P ratio greater than the minimum positive value outlined in the kit instructions for the third and final cell culture passage. If the S/P value for the final cell culture passage is less than the

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minimum positive value, the test is invalid and the original samples should be retested in cell culture as per **Sections 3.6 through 3.9**.

Note: As per Section 3.9, if the testing of any particular series is inconclusive by p27 ELISA, the stored portion of the final harvest may be used as an inoculum for a new test series.

5. Interpretation and Report of Test Results

Results are interpreted as per the instructions supplied with the ELISA kit and the additional test validity requirements outlined in **Sections 4.3.5 through 4.3.7**. The sample is considered negative for ALV p27 antigen if the S/P ratio is less than the minimum positive value listed in the kit instructions. The sample is considered positive for p27 antigen if the S/P ratio is greater than the minimum positive value in the kit instructions. When calculating the S/P ratios, be sure to use the mean absorbance results from the cell controls as the negative control for each of the three respective harvests as per **Section 4.3.6**.

The presence of p27 activity in the harvested samples detected in the second and third harvest is suggestive of ALV replication and these samples are considered positive. Detection of p27 activity in the first harvest is considered to be suspicious; however, if the samples are found negative in the second and third harvest, then the p27 activity found in the first harvest is considered to be non-replicating and the sample is considered negative for extraneous ALV.

5.1 Record the 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

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

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8. Summary of Revisions

Version .03

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

Version .02

- The Contact information has been updated.
- **3.3.2:** Changed Fetal Bovine Serum (FBS) to Newborn Calf Serum or equivalent throughout document.
- **3.5.4:** Deleted 60 mm plastic tissue culture dishes throughout document.
- **3.6.6 and 3.6.7:** Changed Marek's testing to better reflect how testing is currently performed.

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Appendix

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

The following information describes 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.

1. Materials

1.1 Equipment/instrumentation

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

1.1.1 Hemacytometer (1/10 mm deep Neubauer hemacytometer)

1.1.2 Incandescent microscope (capable of 100X magnification)

1.1.3 Hand tally counter

1.2 Reagents/supplies

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

1.2.1 Trypan Blue (0.4%)

1.2.2 Snap cap tube, 6-mL, 12 x 75-mm

1.2.3 Disposable plastic pipettes, 1-, 5-, 10-, and 25-mL

1.2.4 Pipette, 40- to 200- μ L

1.2.5 Disposable pipette tips

2. 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 vortexing. 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

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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 μ L.

| | | |
|---|---|---|
| 1 | | 2 |
| | 5 | |
| 4 | | 3 |

| | | |
|---|----|---|
| 6 | | 7 |
| | 10 | |
| 9 | | 8 |

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 (μ L) to mL (1000). See example below.

$$\begin{array}{r}
 \text{Example: number of cells in 10* squares} = 385 \\
 \text{1:3 cell dilution} = \frac{\times 3}{1155} \\
 \text{cubic mm/mL} \quad \times 1000 \\
 \text{number of cells per mL} = 1155000
 \end{array}$$

* If only 1 chamber is counted (i.e. 5 squares), then take the number of cells in 5 squares x 2 before multiplying by 3 in above example.

3. Formulating cell suspensions

3.1 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:

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

$$DF = 1155000 \text{ cells/mL divided by } 350000 \text{ cells/mL} = 3.3$$

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:

$$375 \text{ mL} / 3.3 \text{ (DF)} = 113.6 \text{ mL}$$

This is the volume of the initial cell suspension needed to make the final volume of cells.

3.3 Finally, take the amount of the initial cell suspension determined in **Section 3.2** 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.

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