



Animal and Plant
Health Inspection
Service

Veterinary Services

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Preparation of Cryopreserved Challenge Cultures for *Leptospira* Serogroups *Canicola*, *Pomona*, *Grippytyphosa*, and *Icterohaemorrhagiae*

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Notes:

Preparation of Cryopreserved Challenge Cultures for Leptospira Serogroups Canicola, Pomona, Grippotyphosa, and Icterohaemorrhagiae

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1. Introduction

This protocol describes the preparation of cryopreserved challenge cultures for *Leptospira* Serogroups Canicola, Pomona, Grippotyphosa, and Icterohaemorrhagiae. The challenges are used in potency testing *Leptospira* fractions in hamsters as outlined in title 9, *Code of Federal Regulations* (9 CFR), parts 113.101 – 113.104. Specific details on the testing protocol performed at the Center for Veterinary Biologics (CVB) are supplied in Supplemental Assay Methods (SAMs) 608, 609, 610, and 617.

2. Composition of the Product

2.1 Strains

Leptospira canicola, CVB Designation 11203

Serogroup Identification: Canicola

REA Identification: *Leptospira interrogans* serovar *Portland-vere*.

Historical Identification: Wood strain or Moulton strain

Leptospira grippotyphosa, CVB Designation 11808

Serogroup Identification: Grippotyphosa

REA identification: *Leptospira kirschneri*. Strain must closely resembles RM52.

Historical Identification: Colony 8 of shrew strain

Leptospira pomona, CVB Designation 11000

Serogroup Identification: Pomona

REA Identification: *Leptospira interrogans* serovar *Kenniwicki* or *Pomona*

Historical Identification: serovar *Kenniwicki* strain MLS

Leptospira icterohaemorrhagiae, CVB Designation 11403

Serogroup Identification: Icterohaemorrhagiae

REA Identification: *Leptospira interrogans* serovar RGA or Monymusk

Historical Identification: CF1 Strain

2.2 Source and History

Leptospira canicola:

The culture was originally obtained by the National Animal Disease Laboratory (NADL) from Philips Roxane, Inc. on June 19, 1969. The culture was passed through dogs in 1984, re-isolated, and then serially transferred through hamsters or stored in liquid nitrogen.

Leptospira grippityphosa:

The culture was originally isolated from a shrew trapped in the northwest United States. It was obtained by the NADL from Catherine Sulzer at the Center for Disease Control on June 6, 1972. It was passed through pigs at the NADL and re-isolated on January 22, 1981. After re-isolation, it was maintained in liquid nitrogen or serially transferred through hamsters at the NADL or CVB.

Leptospira pomona:

The culture was originally identified by Cornell University and then obtained by Philips Roxane, Inc. Prior to April 1969, Philips Roxane, Inc., transferred it to the NADL. The culture was used to challenge calves by the NADL and then re-isolated 72 hours post-inoculation from blood on June 23, 1981. The culture was maintained in liquid nitrogen or serially transferred through hamsters at the NADL and the CVB after this re-isolation.

Leptospira icterohaemorrhagiae:

The culture was originally isolated from a dog in Puerto Rico. It was acquired by Fort Dodge Lab, Inc., in 1958 from Colonel MB Starnes, Director of the Division of Veterinary Medicine at Walter Reed Army Institute of Research. The NADL obtained the culture for Fort Dodge Lab, Inc., in 1969. It was passed through puppies on June 17, 1980, and re-isolated. The culture was maintained in liquid nitrogen or serially transferred through hamsters at the NADL or CVB after this re-isolation.

2.3 Equipment, Instruments, Reagents/Supplies

Equivalent equipment, instrumentation, reagents, or supplies may be substituted for any brand name listed below. Serogroup specific supplies are recommended when reasonable.

- 2.3.1** Animal Biosafety Cabinets (ABSC); two highly recommended
- 2.3.2** Dissection tray
- 2.3.3** Balance, analytical
- 2.3.4** 70% (v/v) ethanol (ETOH), >5 gallon for *L. icterohaemorrhagiae* and *L. grippityphosa* collections, sufficient quantities for sterility for others
- 2.3.5** Small pan, used for ETHO disinfection bath for euthanized hamsters
- 2.3.6** Dissecting pins, 1- to 1 1/2-inch
- 2.3.7** Sterile scalpels
- 2.3.8** Sterile forceps, 5 1/2-inch, rat-tooth or smooth-ended, 2 per pack
- 2.3.9** Petri dishes, 100 mm X 15 mm

- 2.3.10** Syringes, 3 mL with 18 g. 1 1/2" attached needles
- 2.3.11** Biosafety Safety Cabinet (BSC) in BSL2 space
- 2.3.12** Centrifuge and adaptors for 50 mL conical tubes
- 2.2.13** Cotton gauze sponges soaked in 70% (v/v) ethanol
- 2.3.14** Stomacher[®] blender and 18 oz. sterile blender bags, e.g., Whirl-Pak bags, (alternatively, tissue grinders, 15-mL, TenBroeck, may be used)
- 2.3.15** Microscope with darkfield capability; two preferred
- 2.2.16** Microscope slides and cover slips
- 2.2.17** Pipettes, assorted sizes, cotton-plugged
- 2.2.18** Conical tubes, 50 mL polypropylene
- 2.2.19** Erlenmeyer flasks or beakers, 500 mL - 2 L glass, sterile
- 2.2.20** Magnetic stir bars, sterile
- 2.2.21** Magnetic stir plates
- 2.2.22** Sterile personal protective equipment (PPE) including gown, gloves, hairnet, etc.
- 2.2.23** Handi-step[®] with 25 mL sterile positive pressure displacement tips
- 2.2.24** Dry ice (for *L. icterohaemorrhagiae* and *L. grippityphosa* collections) sufficient to fill four 10.5" X 9.5" X 11.5" storage containers
- 2.2.25** Plastic containers (for dry ice)
- 2.2.26** $\leq -72^{\circ}\text{C}$ Freezer (for *L. pomona* and *L. canicola* collections)
- 2.2.27** Liquid nitrogen cell storage freezer(s) with vapor phase storage and appropriate cryocontainers
- 2.2.28** P80-BA STAF+ media
- 2.2.29** 1% Bovine Serum Albumin (BSA)
- 2.2.30** Disposable hemacytomter, Nexcelom Cellometer[®] CPT2-002

2.2.31 Cryovials, 2.0 mL, autoclavable

2.2.32 Cryogenic labels

2.2.33 50% sterile glycerol

2.3 Animals

2.3.1 Hamsters, adult, 50-90 g. Sixty to ninety hamsters per serogroup for final challenge are recommended for the procedure described here. Additional hamsters for three serial passages prior to the cryopreserved challenge should also be available.

2.3.2 The hamsters must be obtained from the same source and colony. All male or all female hamsters are preferred.

2.3.3 House and feed all hamsters in an identical manner.

2.4 Media

The media listed below are recommendations based on current manufacturing lots. Equivalent media is acceptable. Media requirements may alter for future production lots depending on the supplier availability and base media composition changes that are outside the control of CVB-Policy, Evaluation, and Licensing, Bacteriology (CVB-PEL BACT). Endotoxin free glassware designated for *Leptospira* use should be used in media preparation. Quality control issues have been noted when older or common use glassware is used. All media should undergo appropriate growth promotion and sterility testing for quality control.

2.4.1 1% Bovine Serum Albumin Diluent – National Centers for Animal Health (NCAH) Media #20133

Sodium phosphate, dibasic	0.664 g
Potassium phosphate, monobasic	0.087 g
Bovine serum albumin, fraction V	10 g
Deionized water	q.s. 1.0 L

Mix until dissolved. If necessary, adjust pH to 7.5 ± 0.1 . Aliquot 9 mL/glass vial. Sterilize by filtration, using a 0.22- μ m filter. Store at 20°- 25°C for no longer than 1 year.

2.4.2 P80-BA STAF+ medium – NCAH Media #50116

Sodium phosphate, dibasic	0.664 g
Potassium phosphate, monobasic	0.087 g
Sodium chloride	1.925 g
Ammonium chloride	0.268 g
Magnesium chloride	0.191 g

Deionized water 790 mL

Stir to dissolve. Add:

Cupric sulfate solution (300 mg/L, pH 5.8)	1 mL
Zinc sulfate solution (0.4g/L, pH 6.3)	10 mL
Ferrous sulfate solution (2.5 g/L)	20 mL
L-cystine	0.2 g

Stir. Do not attempt to dissolve L-cystine completely. Do not heat.
Filter through triple thickness #1 Whatman paper. If filtrate is not clear, filter again.

Combine filtered media with:

Vitamin B12 solution (10 mg/L)	20 mL
Thiamine HCl solution (2 g/L, pH 3.8)	0.1 mL
Tween 80	1.2 mL
Deionized water	q.s. 1 L

Place 800 mL of this mixture in a large container and autoclave at 121°- 125°C for 20 to 25 minutes. Cool to 56°±1°C.

Combine the following:

Bovine serum albumin, fraction V	20 g
Sodium phosphate, dibasic	0.133 g
Potassium phosphate, monobasic	0.017 g
5-Flourauracil (50 mg/mL)	5 mL
Trimethoprim (20 mg/mL)	1 mL
Sulfamethoxazole (40 mg/mL)	1 mL
Deionized water	q.s. 200 mL

Adjust pH to 7.2 to 7.4 and sterilize by filtration (0.2 µm). Add filtered albumin solution to the cooled (56°C) solution prepared previously.

Adjust to pH 7.2 to 7.8 with sterile 10% NaOH. Dispense in 9 mL aliquots into Glass screw-top tubes, 20 x 150-mm (or equivalent container). Store, tightly capped, at 20°- 25°C for no longer than 6 months.

3. Cultures

3.1 Challenge

3.1.1 Virulent challenge should be serially transferred through hamsters at least three times prior to the week of reagent collection following procedures described in CVB-WI-0253, *Leptospira Passage through Hamsters*.

a. The original challenge inoculum placed into hamsters may be dinger zone from P-80 semi-solid media or cryopreserved homogenate from a previous collection. Challenge inoculums for subsequent passages are infected liver homogenate diluted in 1% BSA.

3.1.2 Select a clinically ill (preferably moribund) hamster from a group of hamsters that were previously infected.

a. Clinical signs indicative of infection include poor coat, severe lethargy, neurologic ataxia, and bloody urine. Bloody nostrils do not correlate well to spirochete load in the organs.

3.1.3 Euthanize the hamster with CO₂. Follow the euthanasia procedure approved by the Animal Care and Use Committee.

3.1.4 Inside a biosafety cabinet, pin the dead hamster to the correctly color-coded dissection board (ventral aspect up). Disinfect the skin with 70% ethanol. Double-check the color marking on the hamster to verify the correct serogroup. Color identifiers are as follows:

- a) *L. canicola*: Green
- b) *L. grippotyphosa*: Purple
- c) *L. icterohaemorrhagiae*: Yellow
- d) *L. pomona*: Red

3.1.5 Using aseptic technique, reflect the abdominal skin with a sterile scalpel and tweezers. Discard the instruments used to open the skin or place in an autoclavable pan for later decontamination.

3.1.6 Using sterile instruments, reflect the abdominal musculature to expose the abdominal viscera. Discard the instruments used to open the abdomen or place in an autoclavable pan.

3.1.7 Using fresh instruments, aseptically remove approximately 1 gram of liver tissue. Aseptically place the liver in a sterile 18 ounce sterile blender bag.

3.1.8 Add 9 mL of sterile 1% BSA diluent to the bag. Twirl or clamp the bag to close and place in the Stomacher. Thoroughly homogenize the liver for 120 seconds, taking care to avoid foam formation. This suspension is considered the 1:10, i.e., 10⁻¹, dilution for this step and similar ones later in the protocol.

3.1.9 Prepare additional serial tenfold dilutions; e.g., 1.0 mL suspension + 9.0 mL diluent, as needed for **Step 3.1.11**. All dilutions should be performed with 1% BSA diluent in glass serum vials. A 3 cc syringe with attached 18 g 1 ½” needle is routinely used to make the serial dilutions.

- a. Scale each dilution based on the total volume required for **Step 3.1.11**.
- b. Hold the dilutions at room temperature (20°- 25°C) and complete challenge inoculations within 2 hours of dilution preparation.

3.1.10 Place 1-2 drops of the 10⁻³ dilution on a microscope slide, cover with a coverslip, and examine under 200X magnification with a darkfield microscope. The 10⁻³ dilution should have at least 30 organisms per field.

- a. Historically, *L. icterohaemorrhagiae* has the highest counts with greater than 80 spirochetes per field. On the other hand, *L. pomona* historically has the lowest counts of the four serogroups.
- b. If the 10⁻³ dilution contains less than 30 spirochetes per field, select another clinically ill hamster and prepare another challenge inoculum that more closely matches the desired organism density. If the desired organism density cannot be obtained after all clinically ill hamsters have been necropsied, select the dilution series with the highest spirochete count and use it to perform one or more serial passage prior to the large scale challenge.

3.1.11 Hamsters should be challenged IP with liver homogenate. A Monday challenge is recommended. Recommended challenge dosages for the hamsters are listed in following table to achieve acceptable *Leptospira* counts in sufficient hamsters on the day of harvest. Biologic variability may occur in response to challenge so a variety of dilutions and inoculum volumes often yields a higher percentage of animals with a high spirochete load than fine-tuning a specific challenge based on a previous outcome(s).

Table 1. Suggested challenge inoculation procedure to reliably yield 1000 cryovials.

	Dilutions					
	10-2		10-3		10-4	
	mL/hamster	Number Innoculated	mL/hamster	Number Innoculated	mL/hamster	Number Innoculated
Canicola			0.5	45	0.75	45
Icterohaemorrhagiae	0.25	15	0.25	15		
	0.75	15	0.75	15		
Pomona	0.5	20	0.75	35	0.5	35
Grippityphosa	0.75	45				
	0.25	45				

4. Harvest

Processing 35 – 55 whole livers with acceptable counts is recommended to yield approximately 1000 cryovials. *L. icterohaemorrhagiae* requires the fewest livers among the serogroups. Higher spirochete counts in the final product are always preferred.

Critical Control Points:

- The time from the first necropsy until the cryovials are placed in $\leq -72^{\circ}\text{C}$ or the dry ice-ethanol slurry must not exceed 16 hours. Spirochetes cannot survive overnight exposure to liver homogenate without being frozen.
- Sterile technique should be followed at all times.

4.1 On the third day following challenge, euthanize hamsters according to **Step 3.1.3** in groups of 5 per technician.

- Hamsters deceased at the time of initial examination should not be used for liver collection.
- Two technicians working simultaneously in two ABSCs are recommended.

4.2 After euthanasia, dip each hamster into a 70% ethanol bath for disinfection prior to starting necropsy of the first animal in the group.

4.3 Expose the abdomen as described in **Steps 3.1.5** and **3.1.6**.

4.4 Aseptically dissect the entire liver and move to a sterile petri dish on a calibrated analytical balance. Carefully remove and discard the gall bladder and then record the weight.

4.5 Place the liver in a numbered sterile blender and clamp or twirl to close. Place the sterile blender bag with liver in a transportable secondary container. Grouping the 5 livers of each harvest group into the same secondary plastic bag for organizational purposes may be helpful.

- a. Each hamster liver should be identified to correlate hamster weight, diluent volume, spirochete counts, and inclusion in the harvest pool later in the process.

4.6. Discard the used petri dish and use fresh sterile instruments for each dissection.

4.7. Repeat **Steps 4.1** through **4.5.** for each hamster. Spray down the BSC with 70% ethanol after each group of 5 hamsters and change disposable non-latex gloves before beginning a new set of 5 hamsters.

4.8 After approximately 20 livers are collected, it is recommend to move them to a BSL2 laboratory space for additional processing by a second set of technicians while the remaining livers are collected. The additional livers can be transported to the BSL2 laboratory space as they are collected for most efficient processing.

4.9 Inside a BSC in the BSL2 laboratory, disinfect the exterior of each sterile blender bag containing liver with 70% ethanol. Add 9 mL of P80-BA STAF+ media per gram of liver to the sterile blender bag. This is considered a 10^{-1} dilution.

- a. Calculating diluent volume for each liver on a spreadsheet may be useful.

4.10 Twirl or clamp the sterile blender bag to close and place in the stomacher. Thoroughly homogenize the liver for 120 seconds.

4.11 Pipette the liver homogenate into 50 mL conical tubes labelled to correspond with the sterile blender bag.

4.12 After vortexing the homogenate, prepare serial ten-fold dilutions in the BSC by transferring 1 mL of the liver homogenate sample to 9 mL of 1% BSA in glass tubes with corresponding identification until 10^{-4} is made.

4.13 Estimate spirochete concentration in each liver homogenate sample. Place 20 μ L of the appropriate dilution on a microscope slide, cover with a coverslip, and examine under 200X magnification with a darkfield microscope. Start with the 10^{-4} dilution and, if needed, examine the 10^{-3} dilution. Exact spirochete counts for individual samples are not required.

- a. If the 10^{-4} dilution contains ≥ 20 spirochetes per field, mark the corresponding 10^{-1} liver homogenate sample as first option for additional processing.
- b. If the 10^{-4} dilution contains < 20 spirochetes per field, examine the 10^{-3} dilution.
- c. If the 10^{-3} dilution contains 70-200 spirochetes per field, mark the corresponding 10^{-1} liver homogenate sample as second option for additional processing.
- d. If the 10^{-3} dilution contains 30-70 spirochetes per field, mark the corresponding 10^{-1} liver homogenate sample as third option for additional processing.

- e. If the 10^{-3} dilution contains < 30 spirochetes per field, the corresponding 10^{-1} liver homogenate sample should be discarded.

4.14 Select all first option 10^{-1} liver homogenate samples for additional processing. If fewer than 50 first option samples are available, add samples from the second option from highest counts to lowest counts. If fewer than 45 first and second option samples are available, add samples from the third option from highest counts to lowest counts.

4.15 Centrifuge the selected 10^{-1} liver homogenate samples in the 50 mL conical tubes at approximately $300 \times g$ for 3 minutes.

4.16 Inside a BSC, aseptically remove the supernatant with a pipette taking care not to disrupt the pellet. Pool the supernatant into a sterile Erlenmeyer flask or beaker. The pellet may be discarded.

- a. The pellet is removed because it increases the viscosity of the final product which, in turn, is painful to the hamsters upon injection.

4.17 Repeat **Step 4.12** for the homogenate pool rather than individual liver homogenates. The homogenate pool must contain at least 30 spirochetes per field under 200X darkfield magnification at the 10^{-3} dilution.

- a. Optional: Up to an equal volume of P80-BA STAF+ media may be used to dilute the pooled liver homogenate if 30 or more viable spirochetes per field are visible under 200X darkfield magnification at the 10^{-4} dilution.
- b. The final spirochete count per mL should be determined using a disposable hemacytometer. Additional tenfold dilutions may be needed for the final spirochete count.

4.18 50% sterile glycerol should be added to a pool of *L. canicola* or *L. icterohaemorrhagiae* to reach a final concentration of 2.5% glycerol. No glycerol should be added to a pool of *L. grippityphosa* or *L. pomona* homogenate.

4.19 Add a sterile magnetic stir bar to the pooled *Leptospira* homogenate.

5. Preparation and Packaging of the Product

5.1 New sterile gowns, gloves, and apparel should be donned prior to filling the reagent vials. The BSC should be emptied and disinfected with 70% ethanol prior to filling the cryovials.

5.2 Place the pooled *Leptospira* homogenate on a stir plate in the BSC throughout the fill.

5.3 Cryovials should be placed in microcentrifuge tube racks and the cryovial lids placed in glass beakers, autoclaved, and allowed to cool prior this step. Both are now placed in the BSC and unwrapped for use.

5.4 Dispense 1.25 ± 0.1 mL of pooled culture per 2.0 mL cryovial. A Handi-Step with 25 mL positive displacement tip may be used. Place sterile cryovial lids on each after fill.

5.5 Vials should be labeled as follows with cryogenic labels:

IRP _____
[Leptospira Name - CVB No]
1.25 mL CVB-PEL [Date]

5.6 Labelled cryovials should be frozen according to the following protocols:

5.6.1 *L. pomona* and *L. canicola*:

- a.** Place cryovials at $\leq -72^{\circ}\text{C}$ for 12 to 20 hours.
- b.** After the overnight freeze, move cryovials to the final liquid nitrogen vapor phase. Containers holding cryovials should be maintained on dry ice if outside a freezer or liquid nitrogen tank for longer than a minute.

5.6.2 *L. icterohaemorrhagiae* and *L. grippityphosa*:

- a.** In a well-ventilated area, create a slurry of dry ice and 70% ethanol. Placing the large storage containers containing the ethanol-dry ice slurry on a rolling cart may be useful to move vials from the initial processing location to the liquid nitrogen tanks. Allow slurry at least 10 minutes to equilibrate before use. The slurry should be just fluid enough to accommodate **b.**, but pieces of dry ice should be visible. Add additional dry ice or ethanol before proceeding or during the procedure to maintain proper conditions.
 - i.** The large storage containers used may vary in size, but a 27" X 16" X 7" container is reasonable.
 - b.** The cryovials should be placed in a floating containment device so they are in an upright position yet still able to be exposed to the dry ice slurry. Cryovials should be submersed (including the caps) in the slurry for 20 minutes to 2 hours. This is referred to as the flash-freeze later in the protocol.
 - i.** Examples of containment devices useful to hold the cryovials during the flash freeze include a laboratory floating rack or the inside divider for a cryogenics box.
 - ii.** Cryovials should be flash-frozen as soon as possible after labelling. Holding cryovials in the flash-freeze is preferred to holding at room temperature.

- c. After flash-freezing, move cryovials to a cryostorage container on dry ice. Each cryovial should have excess slurry from the flash-freeze quickly removed at the time of transfer into the final cryostorage container.
- d. Containers holding cryovials should be maintained on dry ice if outside a freezer or liquid nitrogen tank for longer than a minute.
- e. Place the final cryostorage container in the final liquid nitrogen vapor phase.

CAUTION: Storage or use of dry ice in a poorly ventilated area, particularly a walk-in cooler, can result in asphyxiation. Dry ice storage containers require hazard communication labelling.

5.7 The cryovials should be maintained in liquid nitrogen vapor phase at -130°C or colder for long-term storage.

Table 2: Overview of cryopreservation protocol for each serogroup.

Serogroup	Glycerol (%)	Initial Freezing Conditions	Time for Initial Freeze
Canicola	2.5	≤ -72°C	12-20 hrs
Icterohaemorrhagiae	2.5	Flash-freeze	20 – 120 min
Pomona	0	≤ -72°C	12-20 hrs
Grippityphosa	0	Flash-freeze	20 – 120 min

6. Testing

6.1 Purity test: The harvested *Leptospira* is a primary culture that is unlikely to be completely free of all bacteria found in the hamster environment. However, *Leptospira* should preferentially proliferate faster when returned to the hamster than other bacteria. After three serial passages in hamsters, other bacteria that could complicate potency testing results must not be present in the liver homogenate. Other serogroups of *Leptospira* must not contaminate the cryopreserved material. Additional extraneous agent testing may be recommended as new information and techniques arise.

6.1.1 Bacterial Extraneous Agents:

- a. Cryopreserved reagent:
 - i. 100 µL of cryopreserved reagent is cultured on blood agar plates (BAP) in duplicate for 5 days. Growth present is tested according to 6.1.2.
- b. Challenge post-hamster inoculation:
 - i. At the third serial transfer in hamster and the time of challenge (if additional transfer(s) occur), 100 µL of liver homogenate at the 10⁻⁴

dilution must be cultured in duplicate on BAP for 72 hours. No growth may be present.

6.1.2 Identification of Non-leptospiral Bacteria:

- a. One colony of each type grown directly from the cryopreserved material must be analyzed by MALDI-TOF or comparable technology. None of the bacteria cultured on BAPs should be a pathogenic concern.
 - i. Library databases often do not include non-pathogenic species so an exact identification may be impossible. Genus and species identification is highly preferred to best assess the bacteria.
 - ii. Evidence of non-pathogenicity include: low numbers of individual organisms, mixture of species, and lack of an established pathogenic match in a well-established database.

6.1.3 Viral Contamination:

- a. The cryopreserved reagent must test negative for extraneous viral agents of hamster origin based on hemadsorption testing and hematoxylin and eosin staining on baby hamster kidney (BHK-21-C) cells.
- b. Testing for specific bovine agents may be advised depending on the clearance procedures for the FBS in the P80-BA STAF+ medium.

6.2 Leptospira Identification: Assessment by darkfield microscopy, ELISA, and Microscopic Agglutination Test (MAT) are required. Additional testing such as whole genome sequencing or pulse-field gel electrophoresis should be utilized, if possible, as technology advances. Information on the virulence profile of the organism, such as capacity for conjunctival transmission, may be useful for biosafety profile and is recommended at a later date. Host animal specificity for these strains may be also be valuable to know the reagents utility in efficacy studies and should be considered at a later date.

6.2.1 Darkfield Microscopy

- a. Acceptable spirochete morphology and concentration is required in the serial dilutions of the 10^{-1} pooled liver homogenate prior to the reagent fill and in serial dilutions of each of the infected liver homogenates used for the vaccination-challenge testing of the final product. Spirochetes of normal morphology (10 – 20 μm long) should be visualized under darkfield microscopy. Spirochetes must be predominately mobile and uniform in size. Variability in size and a high proportion of long spirochetes are correlated with decreased virulence. At least 30 spirochetes per field at one dilution between 10^{-3} and 10^{-8} should be visible at 200X magnification during darkfield microscopy.

6.2.2 ELISA

a. An identity and cross-reactivity ELISA is required for an aliquot of final product and infected liver homogenate for each vaccination-challenge assay. The current version of CVB-TWS-0048, *Leptospira ELISA Screen*, should be followed. The appropriate serogroup must be reactive and all other serogroups should be non-reactive.

6.2.3 MAT

a. Serogroups should test positive by MAT according to the serogroup. The tested culture should have no to low cross-reactive titers ($\leq 1:200$) to other serogroups and have a high agglutination titer (1:800) to the appropriate serogroup. One MAT must be performed on challenge from liver homogenate for one of the vaccination-challenge regulatory tests conducted in **Step 6.4.2**. An inconclusive MAT should not discredit the other test results.

i. *Leptospira* serogroup pomona historically has the weakest agglutination. A positive identification on ELISA and a valid hamster vaccination-challenge assays with serogroup specific reference should be used in conjunction with the MAT results.

6.3 Potency:

6.3.1 Transfer Capacity:

a. Four hamsters should be inoculated with 1 mL each of final product. At least 50% of the hamsters must succumb to disease (death or moribundity) to leptospirosis within 7 days of inoculation. *Leptospira* must be successfully transferred at least three times as described in CVB-WI-0253, *Leptospira Passage through Hamsters*. Assessment of transfer capacity may be performed in preparation of **Step 6.3.2**.

6.3.2 Regulatory Testing:

a. Cryopreserved challenge is evaluated by SAMs 608, 609, 610, and SAM 617 as approximately outlined in 9 CFR 113.101 - 113.104. The back-titration hamsters may be eliminated in accordance with Veterinary Services Memorandum No. 800.102. At least three regulatory tests per serogroup must be performed. The regulatory testing must be divided between at least two technicians.

6.4 Stability testing: Regulatory testing according as described in **Step 6.3** should occur at least annually and preferably twice per year per serogroup. Assessment by darkfield microscopy and ELISA identity testing must be conducted in conjunction with the regulatory testing.

7. References

- 7.1 SAM 608, Supplemental Assay Method for Potency Testing of *Leptospira interrogans* serogroup Pomona Bacterins
- 7.2 SAM 609, Supplemental Assay Method for Potency Testing of *Leptospira interrogans* serogroup Canicola Bacterins
- 7.3 SAM 610, Supplemental Assay Method for Potency Testing of *Leptospira interrogans* serogroup Icterohaemorrhagiae Bacterins
- 7.4 SAM 617, Supplemental Assay Method for Potency Testing of *Leptospira kirschneri* serogroup Grippityphosa Bacterins
- 7.5 BBWI0107, *Leptospira* Passage through Hamsters
- 7.6 BBTWS0214, *Leptospira* ELISA Screen
- 7.7 Faine, Solomon. *Leptospira and Leptospirosis*. CRC Press. Boca Raton, Florida. 1994.

8. Summary of Revisions

Version CVB-PP-0027.02

- Updated formatting for consistency with current processes.
- In Section 6.2.3, restriction on the group that could perform MAT was removed.