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**CSS MINIMUM REQUIREMENTS
FOR
DISEASE CONTROL OF SEMEN PRODUCED FOR AI**

The "CSS Minimum Requirements for Disease Control of Semen Produced For AI" provides a minimum standard for the health monitoring and disease surveillance of bulls prior to entering isolation, during an isolation period and throughout residency at an AI center. This is a comprehensive standard for those diseases proven to be a significant threat to be seminally transmitted by artificial insemination. Furthermore, it outlines proper sanitary procedures and includes requirements for the addition of appropriate antibiotics to semen and extender to control specific microorganisms. The goal of these requirements is to protect the health of the seminal donors and the herds in which the semen is used.

GENERAL SANITARY CONDITIONS

1. Semen collection equipment which comes in contact with the bull or his secretions or excretions shall be thoroughly disinfected after each use.
2. New disposable plastic gloves shall be used by the collector on each bull to assure that his hands cannot serve as a means of transmitting infectious, contagious material from bull to bull.
3. The laboratory used for semen processing shall be fully enclosed and partitioned from bull housing and semen collection areas, and structured to provide for hygienic handling and storage of semen.
4. The health tests to be conducted in accordance with the following requirements shall be conducted in a manner generally consistent with the procedures described in "The Recommended Uniform Diagnostic Procedures for Qualifying Bulls for the Production of Semen" as published by the American Association of Veterinary Laboratory Diagnosticians (AAVLD) or other diagnostic procedures recognized as being at least equal to the AAVLD published procedures.
5. Attention shall be given to liquid nitrogen refrigerators returning from foreign countries not declared free of foot and mouth disease by USDA, to determine if they have been disinfected at the port of entry. If they have been properly disinfected, there will be a tag attached indicating this fact. If disinfection has not been done, the USDA/APHIS veterinarian in the state involved shall be notified and appropriate action shall be taken immediately to have the refrigerators properly disinfected.

MOUNT ANIMALS

Mount animals (teasers) used during semen collection shall be submitted to the same regimen of periodic health tests as bulls in semen production and be maintained continuously in a health testing status equivalent to the CSS bulls. Mount animals shall not be interchanged between the CSS resident herd and the CSS isolation testing environments. Areas of contact by the erect penis or of genital secretions upon the hair coat or skin of a mount shall be effectively and thoroughly disinfected between successively mounting bulls.

PRE-ENTRY TO ISOLATION

Bulls and mount animals that are intended to enter a CSS-approved AI Center shall be healthy and free of infectious or contagious diseases and shall not originate from a herd under quarantine. Subsequent to the pre-entry testing described below, the bulls and mount animals should not be used for natural service and should be isolated from other cattle. Isolation means no direct contact or fence line contact with other cattle.

The following pre-entry examination and diagnostic tests shall be conducted and results received for each bull and mount animal prior to commencing the isolation interval. These tests are preferably conducted prior to arrival at the isolation facilities of the AI Center. However, these tests may be conducted in a separate facility at the AI Center, as described below, but the animal isolation interval shall not commence until results of the pre-entry tests are known.

For purposes of these requirements, pre-entry testing performed at the AI Center shall mean that bulls and mount animals must be housed in a pre-isolation facility that is effectively separated from facilities occupied by resident bulls and mount animals, and also separate from bulls and mount animals housed in isolation facilities. All equipment used to handle bulls and mount animals for semen collection, feeding, watering, and cleaning in isolation or resident herds shall not be used at the pre-isolation facility.

1. Physical Examination: A physical examination shall be conducted by an accredited veterinarian within 30 days prior to entry to determine that the bulls or mount animal do not display any clinical symptoms of any infectious, contagious disease.
2. Tuberculosis: An intradermal tuberculin test shall be conducted within 60 days prior to entry; the result shall be negative.
3. Bovine Brucellosis: A buffered brucella antigen test (Card or BAPA) or a complement fixation (CF) test shall be conducted within 30 days prior to entry; the result shall be negative. The brucellosis test should comply with applicable regulations if the animal must be transported interstate.
4. Bovine Leptospirosis: A blood test for serotypes L. pomona, L. hardjo, L. canicola, L. icterohaemorrhagiae, and L. grippityphosa shall be conducted within 30 days prior to entry. Any animal with a significant titer may be subjected to a second blood test within two to four weeks after the first. An end or limiting titer (1:100 or greater) may be run on both samples. Cattle with a stabilized low titer (negative at 1:400) on both tests may be considered satisfactory to enter the isolation facility.
5. Bovine Viral Diarrhea Virus (BVDV): A blood test for BVDV shall be conducted within 30 days prior to entry; the result shall be negative. The test for BVDV shall be a viral isolation test of whole blood or serum (see ISOLATION, 1., f., ii.) performed in bovine cell culture followed by staining of the cell culture by immunofluorescence (FA) or immunoperoxidase (IP) methods, **OR** an antigen capture ELISA, **OR a PCR test**.

ISOLATION

Each bull and mount animal shall be held in isolation throughout the period of time necessary to conduct the tests listed below. Each bull and mount animal shall successfully complete the isolation protocol before being permitted to enter the facilities occupied by resident bulls and mount animals and before any semen from the bull is released for use.

For purposes of these requirements, isolation shall mean that the bulls and mount animals are housed in facilities under the control (supervision) of the AI company. These facilities are effectively separated from facilities occupied by resident bulls and mount animals and all equipment used to handle the bulls and mount animals for semen collection, feeding and watering, and cleaning the facilities occupied by the bull or mount

animal shall not be used for both isolation and resident herds. Further, semen collection areas for bulls in isolation shall be effectively separated from areas used for resident bulls.

1. The following tests shall be conducted on all bulls and mount animals while resident in the isolation facility.

- a. Tuberculosis: One intradermal tuberculin test; the result shall be negative. This test shall be conducted at least sixty (60) days after the date of a pre-entry test for tuberculosis.
- b. Bovine Brucellosis: One buffered brucella antigen test (Card or BAPA) and one complement fixation (CF) test with negative results. These serological tests shall be conducted not sooner than thirty (30) days after the date of the pre-entry test for brucellosis.

Should the bull have a result other than negative, it is recommended that another official USDA brucellosis test be conducted. A negative result on retest or on additional official brucella tests may permit the bull a negative brucella classification, but final classification remains the prerogative of the state veterinary officials.

- c. Bovine Leptospirosis: serological test for serotypes L. pomona, L. hardjo, L. canicola, L. icterohaemorrhagiae, and L. grippityphosa. This test shall be conducted not sooner than thirty (30) days after the date of the pre-entry tests for leptospirosis. A negative result is preferred. However, if the result is not negative (that is positive at 1:100 or higher), the bovine must have at least one retest conducted at least 14 days following the previous test. Cattle that are negative at 1:400 on at least two consecutive tests are considered to have a stabilized low titer.
- d. Bovine Campylobacteriosis: Preputial material shall be cultured and examined for *Campylobacter fetus venerealis*, the result shall be negative. As an alternative procedure, the preputial material may be examined using the fluorescent antibody (FA) technique as a screening test. Any positive FA test shall be followed by a culture of preputial material, the result shall be negative.

Bulls and mount animals may be placed on the following variable testing schedule:

<u>Age of sire when entering isolation</u>	<u>Minimum number of tests (at weekly intervals)</u>
Less than 180 days *	1
180 – 364 days	3
365 days and over	6

* Providing AI center veterinarian can certify that bull has not been housed with female cattle since reaching the age of thirty (30) days.

- e. Bovine Venereal Trichomoniasis: Microscopic examinations of cultured preputial material collected from the fornix shall be negative. The frequency of testing shall be the same as that listed under ISOLATION 1.d. Bovine Campylobacteriosis.
- f. Bovine Viral Diarrhea Virus (BVDV): All bulls and mount animals entering CSS approved AI centers must be tested for viremia ~~to~~ and persistent BVDV infection **while in isolation**, with negative results before entry into the AI Center's resident herd. **Testing is to be accomplished no sooner than 10 days after entry into the isolation facility.** Furthermore, all bulls are to be evaluated by a testing program to detect persistent testicular infection.

The following test methods and schedules are to be used to test for persistent BVD viremic infection.

- i. Diagnostic test: The animal must be subjected to a **PCR test on whole blood or a virus isolation test with one pass** performed in bovine cell culture with a negative result as demonstrated by staining of the cell culture by immunofluorescence (FA) or immunoperoxidase (IP) methods.
- ii. Diagnostic specimens: **PCR test on whole blood. Virus isolation on** either whole blood or serum, **but whole blood must be used for animals less than 6 months of age.**
- iii. Confirmation of persistent BVDV infection: If BVDV is demonstrated by FA or IP in cell culture **OR by PCR**, the animal is to be isolated from other cattle and retested in not less than 21 days by **PCR (serum in this case, regardless of the age of the animal) OR** inoculation of bovine cell cultures with an appropriate specimen (whole blood or serum **depending on the age of the animal**). Demonstration of BVDV a second time is considered confirmation of persistent infection and the animal is not eligible to enter the resident herd of the CSS-approved AI center.
- iv. Confirmation that an animal is not persistently infected: Animals from which BVDV has been isolated or demonstrated must remain in isolation apart from other cattle until proven free of BVDV by 2 consecutive negative virus isolation tests conducted at least 10 days apart and performed on the appropriate specimen (whole blood or serum).

Bulls from which BVDV has been isolated but are later proven to be free of persistent infection (as stated above in iv.) must have samples of any semen that were collected and processed within the 30 days preceding and following the date of positive virus isolation, subjected to BVDV isolation tests **or PCR** with negative results from each collection code before distribution.

2. The following test shall be conducted for all bulls before their semen is released. If the bulls are not of semen producing age during the CSS isolation period, this test may be conducted after the isolation period is completed:

Bovine Viral Diarrhea Virus (BVDV): One of the following test methods and schedules is used to test for persistent testicular BVDV infection.

- i. Test all bulls anytime during the isolation interval for BVDV by the serum neutralization (SN) test **for both types I and II**. All bulls that test positive must have one **negative PCR test on processed semen, or a virus isolation test on processed semen with one pass** performed in bovine cell culture with a negative result as demonstrated by staining of the cell culture by immunofluorescence (FA) or immunoperoxidase (IP) methods. Processed semen is semen that is completely extended and frozen.

- OR -

- ii. All bulls must have one **negative PCR test on processed semen or a virus isolation test with one pass** of processed semen performed in bovine cell culture with a negative result as demonstrated by staining of the cell culture by immunofluorescence (FA) or immunoperoxidase (IP) methods. Processed semen is semen that is completely extended and frozen.

[Any bulls with a positive virus isolation test of semen should have additional processed semen tested to confirm persistent testicular infection.]

Note: Any bull that has a persistent testicular infection for BVDV is not eligible for semen collection and is not permitted to remain in the resident herd.

3. All semen shall be treated with the antibiotics gentamicin, tylosin and Linco-Spectin (GTLS) as described by Shin, et al (1) Lorton, et al (2) and Lorton, et al (3). Details of the procedures to be used are listed in Appendix 1.

RESIDENT HERD

Once a bull or mount animal has completed the isolation testing outlined above, he may enter the resident herd where he shall continue to be tested in accordance with the below listed test procedures.

1. The following tests shall be conducted for all bulls and mount animals at six (6) month intervals:
 - a. Tuberculosis: The official intradermal tuberculin test, with negative result.
 - b. Bovine Brucellosis: One buffered brucella antigen test (Card or BAPA) and one complement fixation test with negative results. If result of either test is not negative, refer to ISOLATION 1. b. Bovine Brucellosis for additional information.
 - c. Bovine Leptospirosis: Serological test for serotypes L. pomona, L. hardjo, L. canicola, L. icterohaemorrhagiae, and L. grippotyphosa. If result is not negative, the bull must have a stabilized low titer. Refer to ISOLATION 1.c. Bovine Leptospirosis.
 - d. Bovine Venereal Trichomoniasis: A single microscopic examination of cultured preputial material with negative result.
 - e. Bovine Campylobacteriosis: A single culture test of preputial material with negative result. As an alternative procedure, the preputial material may be examined using the fluorescent antibody (FA) technique. Any positive FA test shall be followed by culture of preputial material, the result shall be negative.

Antibiotics shall be added to all processed semen as described above (refer to ISOLATION 3.).

2. All bulls or mount animals in the resident herd shall be maintained in continuous isolation from all cloven hoofed animals that have not completed all of the test procedures outlined herein with negative results. At any time that an individual bull or mount animal from the resident tested herd is permitted contact with an untested animal he shall be removed immediately from the resident tested herd and shall not be permitted re-entry until such time as he has completed another cycle of isolation and the tests prescribed therefor, except as provided for in paragraph 3 below.
3. It is not required that a bull temporarily held out of semen production be tested for bovine trichomoniasis and bovine campylobacteriosis provided he is at a location effectively separated from the resident herd. However, he shall be maintained in a herd which otherwise meets all conditions of a resident herd. The routine testing regimen as defined for the resident herd must be resumed prior to the release of semen that was processed after the bull's return to production.

ANTIBIOTICS AND SEMEN PROCESSING

1. Antibiotics will be added to the neat semen and extender to provide effective microbiological control of:

Mycoplasmas
Ureaplasmas
Histophilus somni
Campylobacter fetus subsp. venerealis

2. Effective microbiological control is the condition in which the number of organisms potentially present are reduced to below the threshold of infectivity.
3. An acceptable protocol is the treatment of semen and extender with the antibiotics gentamicin, tylosin, lincomycin and spectinomycin (GTLS) as described by Shin, et al (1) Lorton, et al (2) and Lorton, et al (3). Details of the procedures to be followed are described in, Section I of Appendix 1.
4. Acceptable alternative protocols must provide effective microbiological control (of organisms in 1 above) based on scientific evidence, submitted to Certified Semen Services, Inc. An example of an approved alternative protocol is the 1-step procedure as described by Shin and Kim (4). Details are described in Section II of Appendix 1.

REFERENCES

- (1) Shin, S.J., D.H. Lein, V.H. Patten and H.L. Ruhnke. 1988. A New Antibiotic Combination for Frozen Bovine Semen. 1. Control of Mycoplasmas, Ureaplasmas, Campylobacter fetus subsp. venerealis and Haemophilus somnus. Theriogenology. 29:577.
- (2) Lorton, S.P., J.J. Sullivan, B. Bean, M. Kaproth, H. Kellgren and C. Marshall. 1988. A New Antibiotic Combination for Frozen Bovine Semen. 2. Evaluation of Seminal Quality. Theriogenology. 29:593.
- (3) Lorton, S.P., J.J. Sullivan, B. Bean, M. Kaproth, H. Kellgren and C. Marshall. 1988. A New Antibiotic Combination for Frozen Bovine Semen. 3. Evaluation of Fertility. Theriogenology. 29:609.
- (4) Shin, S.J. and S.G. Kim. 2000. Comparative Efficacy Study of Bovine Semen Extension: 1-Step vs 2-Step Procedure. Proceedings 18th Technical Conf. on Artificial Insemination and Reproduction, NAAB, Columbia, MO. pp. 60 – 62.

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APPENDIX 1

GTLS ANTIBIOTIC PROCEDURES/CONDITIONS

I. Standard CSS Protocol (2-Step Method)

A. Antibiotics/Stock Solutions

(1) Antibiotics:

- a. Gentamicin sulfate: powder, micronized, non-sterile, U.S.P. (veterinary grade), 100 grams per bottle.
- b. Tylosin: labeled as Tylan Soluble, product of Elanco Products Company, 100 grams per bottle.
- c. Linco-Spectin: product of the Upjohn Company, 20 ml per vial, each ml contains 50 mg lincomycin and 100 mg spectinomycin.

NOTE: Antibiotics obtained from some sources have not been tested and may contain deleterious agents that may harm or kill sperm cells. For recommended sources, contact Certified Semen Services.

- (2) Stock solutions of individual antibiotics (gentamicin and tylosin) may be prepared and stored separately at 5°C for eight days or stored frozen in LN vapor for up to six months. Linco-Spectin as supplied by distributor should be maintained at 5°C after it is opened.
- (3) Stock solutions of individual antibiotics will be combined on day of use, and not held over.
- (4) Extenders must be used on the day the combined antibiotics are added.

B. Neat Semen Treatment

- (1) 100 µg of tylosin, 500 µg gentamicin and 300/600 µg of Linco-Spectin dissolved in .02 ml of double distilled sterile water will be added and carefully mixed with each ml of neat semen.

NOTE: All of the antibiotic concentrations expressed herein are for active units of antibiotic. Potency values may vary between batches of antibiotic. Therefore, amounts of raw material have to be adjusted for each batch in order to obtain the required concentrations of active antibiotic.

- (2) The addition of these antibiotics should be scheduled so as to allow a three to five minute time period for the antibiotics to be in contact with the neat semen before the addition of any extender.

C. Non-Glycerol Fraction of Extender

- (1) All non-glycerol fractions of any of the five extenders listed below will be prepared to contain the following concentrations of antibiotics before being added to semen:

tylosin	100 µg per ml
gentamicin	500 µg per ml
Linco-Spectin	300/600 µg per ml

- (2) A volume of this extender (up to 50 percent of the planned final extended volume) is added to the neat semen prior to cooling. All semen must be held in contact with the non-glycerol extender for a minimum of two hours prior to the addition of any glycerol containing extender.

D. Glycerol Containing Fraction of Extender

- (1) This fraction of the extender may contain 5-10 percent of the antibiotic concentration listed under C.(1) Non-Glycerol Fraction of Extender.
- (2) The glycerol fraction of the extender should be added to the non-glycerol fraction of extender plus semen at a 1 to 1 ratio.

E. Final Concentration of Antibiotics

Following the above procedures will yield a final concentration of 50 μg tylosin, 250 μg gentamicin and 150/300 μg of Linco-Spectin in each ml of frozen semen.

F. Required Processing Procedures

It has been shown that processing procedures, extender composition, and antibiotic combinations may affect efficacy of microbial control or fertility. Therefore, deviation from the following may require the organization to conduct additional efficacy testing:

- (1) Use of extender other than one approved by CSS.
- (2) Antibiotic/neat semen contact of less than three minutes.
- (3) Cooling of semen and non-glycerol fraction less than two hours to 5°C.
- (4) Glycerol is not an extender component until after cooling to 5°C.

G. Deviation from Required Processing Procedures

If there is deviation from any of the procedures listed under Section F:

- (1) A written request for an exception will be made to the Service Director of CSS.
- (2) The CSS Service Director will determine whether the deviation will require testing for efficacy. Appropriate efficacy testing may be done at a laboratory approved by CSS that has demonstrated competency for carrying out these analyses.
- (3) The test results will be returned from the laboratory to the CSS Service Director and the requesting organization.
- (4) If the results demonstrate efficacy equal to or greater than obtained by Shin (1) then permission to use the procedure will be granted.
- (5) All fees and expenses for these tests will be paid by the organization making the request.

H. Tested and Approved Extenders

The following five extenders have been tested for efficacy of control of microbial organisms. Use of the antibiotic combination in extenders 1 and 3 did not adversely effect post-thaw motility or fertility (extenders 2, 4, and 5 were not evaluated). In addition to the five extenders listed here other extenders have been approved by CSS through procedures outlined in Section G. ***To determine if an extender not listed here has been approved, contact the CSS Service Director for appropriate information.*** Antibiotics dissolved in double distilled sterile water should be included in the preparation of extenders to yield the final volumes shown under Section I, E of Appendix 1. The final composition of each extender is as follows:

(1) Egg Yolk Citrate

20% Egg yolk
2.12 gm % sodium citrate dihydrate
0.183 gm % citric acid monohydrate
7.0% glycerol

(2) 20% Egg Yolk-Tris

20% egg yolk
2.42 gm % tris (hydroxymethyl aminomethane)
1.38 gm % citric acid monohydrate
1.0 gm % fructose
7.0% glycerol

(3) Heated Whole Milk

7.0% glycerol

(4) Plus-X

Plus-X, as supplied by distributor.
7.0% glycerol

(5) 28% Egg Yolk-Tris

28% egg yolk
1.92 gm % tris (hydroxymethyl aminomethane)
1.10 gm % citric acid monohydrate
1.00 gm % glucose
7.0% glycerol

The following commercially available extenders have been approved for use by CSS:

Biociphos (2-step) - IMV International Corp.
Biladyl (2-step) - Minitube of America, Inc.
Concentrated (2-step) - Continental Plastic Corp.
Concentrated (1-step*) - Continental Plastic Corp.
Viam Pac (2-step) - Viam Pac Inc.
BioXcell (2-step) - IMV International Corp.

BioXcell (1-step*) - IMV International Corp.
BoviPro Cryoguard (2-step) - Minitube of America, Inc.
BoviPro Cryoguard (1-step*) - Minitube of America, Inc.
Triladyl-CSS (1-step*) - Minitube of America, Inc.
Andromed-CSS (2-step) - Minitube of America, Inc.
Andromed-CSS (1-step*) - Minitube of America, Inc.

* 1-step extenders require additional quantities of GTLS antibiotics as described in Section II. They are to be used according to procedures outlined by Shin and Kim (4) and they have not been tested nor approved by CSS for room temperature semen processing. Antibiotics for 1-step extenders must be made up (at time of use) from dry components. Consequently, any antibiotics included in liquefied extender concentrates from the manufacturer should not be considered toward the final required concentrations.

REFERENCES:

- (1) Shin, S.J., D.H. Lein, V.H. Patten and H.L. Ruhnke. 1988. A New Antibiotic Combination for Frozen Bovine Semen. 1. Control of Mycoplasmas, Ureaplasmas, Campylobacter fetus subsp. venerealis and Haemophilus somnus. Theriogenology. 29:577.

II. Alternative CSS Protocol (1-Step Method)

A. General Description

As described by Shin and Kim (4), this processing protocol is approved only for 20% Egg Yolk Tris extender (see Section I, H, 2 of Appendix 1). It requires the same preparation of antibiotics/stock solutions (see Section I, A of Appendix 1); and neat semen treatment (see Section I, B of Appendix 1) as the standard 2-step protocol. However the main differences from the standard CSS 2-step protocol are as follows:

- (1) The extender is not fractionated into a non-glycerol and glycerol component. The complete extender contains 7.0% glycerol.
- (2) The concentration of GTLS antibiotics in each ml of extender is the same as that prescribed for neat semen treatment (i.e., 100 μg tylosin, 500 μg gentamicin, 300/600 μg Linco-Spectin. Thus the final concentration of antibiotics is essentially doubled compared to the standard 2-step protocol.

B. Neat Semen Treatment

Identical to that for the standard 2-step protocol. See Section I, B, 1 and 2 of Appendix 1.

C. Final Concentration of Antibiotics

The 1-step protocol will yield a final concentration of 100 μg tylosin, 500 μg gentamicin, and 300/600 μg of Linco-Spectin in each ml of frozen semen.

D. Required Processing Procedures

It has been shown that processing procedures, extender composition, and antibiotic combinations may affect efficacy of microbial control or fertility. Therefore, deviation from the following may require the organization to conduct additional efficacy testing:

- (1) Use of extender other than one approved by CSS and described by Shin and Kim (4).
- (2) Antibiotic/neat semen contact of less than three minutes.

E. Deviation from Required Processing Procedures

If there is deviation from any of the procedures listed under Section D above:

- (1) A written request from an exception will be made to the Service Director of CSS.
- (2) The CSS Service Director will determine whether the deviation will require testing for efficacy. Appropriate efficacy testing may be done at a laboratory approved by CSS that has demonstrated competency for carrying out these analyses.

- (3) The test results will be returned from the laboratory to the CSS Service Director and the requesting organization.
- (4) If the results demonstrate efficacy equal to or greater than obtained by Shin (1) or Shin and Kim (4) then permission to use the procedure will be granted.
- (5) All fees and expenses for these tests will be paid by the organization making the request.

REFERENCES:

- (1) Shin, S.J., D.H. Lein, V.H. Patten and H.L. Ruhnke. 1988. A New Antibiotic Combination for Frozen Bovine Semen. 1. Control of Mycoplasmas. Ureplasmas. Campylobacter fetus subsp. venerealis and Haemophilus somnus. Theriogenology. 29:577.
- (4) Shin, S.J. and S.G. Kim. 2000. Comparative Efficacy Study of Bovine Semen Extension: 1-Step vs 2-Step Procedure. Proceedings 18th Technical Conf. on Artificial Insemination and Reproduction, NAAB, Columbia, MO. pp. 60-62.

APPENDIX 2

BASIC AI CENTER TESTING PROTOCOL

The basic health testing program is outlined in the "CSS Minimum Requirements for Disease Control of Semen Produced for AI." These requirements have been developed over the years by the AI industry to help ensure semen used in AI is not a vehicle for transmitting those disease agents of concern.

Following is a summary of the CSS testing program:

	TESTING ENVIRONMENTS		
	Pre-entry To Isolation (Within 30 days prior to entering isolation facilities)	Isolation (Testing before entry into a resident herd and semen release)	Resident Herd (Semen collection center)
<u>Physical Examination</u>	Conducted by accredited veterinarian.		
<u>Tuberculosis</u>	Negative intradermal tuberculin test. (Within 60 days prior to entry)	Negative intradermal tuberculin test at least 60 days after pre-entry test.	Negative intradermal tuberculin test at 6 month intervals.
<u>Brucellosis</u>	Official test of state where bull is located. Blood serum test (CF, BAPA or Card).	Complement fixation (CF) and one BAPA or Card test at least 30 days after pre-entry testing.	Complement fixation (CF) and one BAPA or Card test at 6 month intervals.
<u>Bovine Viral Diarrhea Virus</u>	One negative virus isolation test performed on either whole blood (animals less than 6 months of age) or serum, or an antigen capture ELISA, or a PCR test.	One negative virus isolation test performed on either whole blood (animals less than 6 months of age) or serum or a negative PCR test on whole blood, at least 10 days after entry into isolation. Negative virus isolation test or PCR test of processed semen before release for use, or negative virus isolation test or PCR test of processed semen for any donors testing BVDV positive by SN test for types I and II.	
<u>Leptospirosis</u>	Blood test for 5 serotypes important in USA*.	Blood test for 5 serotypes important in USA*. No sooner than 30 after pre-entry test.	Blood test for 5 serotypes important in USA* at 6 month intervals.

TESTING ENVIRONMENTS

Pre-entry To Isolation (Within 30 days prior to entering isolation facilities)	Isolation (Testing before entry into a resident herd and semen release)	Resident Herd (Semen collection center)
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Campylobacteriosis

	<p>Series of negative culture tests of preputial material or screening by fluorescent antibody (FA) with any positive FA tested by culture for final determination.</p> <p>Bulls under 180 days of age - negative on 1 test→.</p> <p>Bulls 180-364 days of age - negative on 3 weekly tests.</p> <p>Bulls 365 days or older tested negative on 6 weekly tests.</p>	<p>Negative single culture test of preputial material or FA for screening test at 6 month intervals.</p>
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Trichomoniasis

	<p>Series of negative microscopic examinations of cultured preputial material.</p> <p>Bulls under 180 days of age - negative on 1 test→.</p> <p>Bulls 180-364 days of age - negative on 3 weekly tests.</p> <p>Bulls 365 days or older tested negative on 6 weekly tests.</p>	<p>Negative single microscopic test of cultured preputial material at 6 month intervals.</p>
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* *L. pomona*, *L. hardjo*, *L. canicola*, *L. icterohaemorrhagiae*, *L. grippotyphosa*

→ Providing AI center veterinarian can certify that bull has not been housed with female cattle since reaching the age of 30 days.

Antibiotic Treatment of All Semen and Extender

- 1) Neat Semen Treatment - 100 µg of tylosin, 500 µg gentamicin and 300/600 µg of Linco-Spectin dissolved in 0.02 ml of double distilled sterile water, added and mixed with each ml of neat semen.
- 2) CSS Approved Semen Extender (Standard 2-Step Method) - The same antibiotics are added to the extender such that the final concentration is 50 µg tylosin, 250 µg gentamicin and 150/300 µg of Linco-Spectin in each ml of frozen semen.
- 3) CSS Approved Semen Extender (Alternative 1-Step Method) - The same antibiotics are added to the extender such that the final concentration is 100 µg tylosin, 500 µg gentamicin and 300/600 µg of Linco-Spectin in each ml of frozen semen.