CERTIFIED SEMEN SERVICES

Approval date: February 25, 2021
Effective date: July 1, 2021

CSS MINIMUM REQUIREMENTS FOR DISEASE CONTROL OF SEMEN PRODUCED FOR ARTIFICIAL INSEMINATION

The "CSS Minimum Requirements for Disease Control of Semen Produced for Artificial Insemination" 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 provide control for specific pathogenic microbial contamination. 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 or discarded if single 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 laboratory health tests are to be conducted in a veterinary diagnostic laboratory (or laboratories) that is AAVDL accredited and approved by the USDA to perform the requested health tests for export purposes. For AI Centers located outside of the USA, health tests are to be conducted in a veterinary diagnostic laboratory (or laboratories) that are approved by the veterinary competent authority of that country to perform the requested heath tests for export purposes.

5. Attention shall be given to cryogenic shipping containers 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 cryogenic shipping containers 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 cleaned and 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 entering the isolation facility and 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 and the bull or mount animal is moved to the isolation facility.

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 or other livestock facilities operated by the AI Center.

1. Physical Examination: A physical examination shall be conducted by a Category II USDA accredited veterinarian (or equivalent in other countries) 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. Bovine Tuberculosis: An official intradermal Caudal Fold Test (CFT) shall be conducted within 60 days prior to entry; the result shall be negative.

   Should the animal have a CFT result other than negative, a confirmatory test with negative result for bovine tuberculosis in accordance with USDA regulations and guidelines is required.

   Note: International bovine semen export regulations vary among countries and not all USDA approved bovine tuberculosis tests are accepted for export.

3. Bovine Brucellosis: A buffered brucella antigen test (Card or BAPA) **OR** a complement fixation (CF) test **OR** a florescent polarization assay (FPA) test on serum shall be conducted within 30 days prior to entry; the result shall be negative.

   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 federal or state veterinary officials.

   The brucellosis test should comply with applicable regulations if the animal must be transported interstate.

4. Bovine Leptospirosis: A microscopic agglutination test (MAT) on serum for serotypes *L. pomona*, *L. hardjo*, *L. canicola*, *L. icterohaemorrhagiae*, and *L. grippotyphosa* with a negative result. This test shall be conducted within 30 days prior to entry with a negative result.

   A negative result is preferred for each serotype. However, if the result is not negative (that is positive at 1:100 or 1:200) for any serotype, the bovine must have at least one retest for that serotype conducted at least 14 days following the previous test. Cattle that are **negative at 1:400** on two consecutive tests have a stabilized low titer and considered negative. Both tests shall be conducted within 30 days prior to entry.

5. Bovine Viral Diarrhea Virus (BVDV): An antigen detection test for BVDV shall be conducted within 30 days prior to entry; the result shall be negative. The test for BVDV shall be one of the following tests on whole blood or serum. A PCR test **OR** an antigen capture ELISA test **OR** A virus isolation test performed in bovine cell culture with a negative result demonstrated by testing the cell culture by PCR or by staining of the cell culture by immunofluorescence (FA) or immunoperoxidase (IP) methods.

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 of the AI company and supervision of a Category II USDA accredited veterinarian (or equivalent for facilities in other countries). 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 unless there is a documented sanitation and disinfection process used on the equipment after each use (logbook and SOP). Further, semen collection areas for bulls in isolation shall be effectively separated from areas used for resident bulls.

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

1. Bovine Tuberculosis: An official intradermal Caudal Fold Test (CFT) shall be conducted at least 60 days after the date of a pre-entry test for bovine tuberculosis and not sooner than 21 days after entering the isolation facility; the result shall be negative.

   Should the animal have a CFT result other than negative, a confirmatory test with negative result for bovine tuberculosis in accordance with USDA regulations and guidelines is required.

   Note: international bovine semen export regulations vary among countries and not all USDA approved bovine tuberculosis tests are accepted for export.

2. Bovine Brucellosis: A buffered brucella antigen test (Card or BAPA) **OR** a complement fixation (CF) test **OR** a florescence polarization assay (FPA) on serum with a negative result. The serological test shall be conducted not sooner than 30 days after the date of the pre-entry test for brucellosis and not sooner than 21 days after entering the isolation facility.

   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 federal or state veterinary officials.

3. Bovine Leptospirosis: A microscopic agglutination test (MAT) on serum for serotypes *L. pomona*, *L. hardjo*, *L. canicola*, *L. icterohaemorrhagiae*, and *L. grippotyphosa* with a negative result. This test shall be conducted not sooner than 30 days after the date of the pre-entry tests for leptospirosis and not sooner than 21 days after entering the isolation facility

   A negative result is preferred for each serotype. However, if the result is not negative (that is positive at 1:100 or 1:200) for any serotype, the bovine must have at least one retest for that serotype conducted at least 14 days following the previous test. Cattle that are **negative at 1:400** on two consecutive tests have a stabilized low titer and considered negative.

4. Bovine Campylobacteriosis and Bovine Venereal Trichomoniasis:

   a. Microscopic examination of cultured preputial material **OR** PCR test of preputial material collected from the fornix of the prepuce for *Tritrichomonas foetus*, with a negative result.

   b. Examination of cultured preputial material **OR** PCR test of preputial material collected from the fornix of the prepuce for *Campylobacter fetus* subsp. *venerealis*, with a negative result.

   Bulls and mount animals shall be placed on the following variable test schedule based on their age on the day of entry into the CSS Isolation facility with testing completed at least at weekly intervals to complete the minimum number of tests, each with a negative result during isolation for both:

Age of sire when entering isolation	**Minimum number of tests**
Less than 180 days with certification* (see below)	1
Less than 180 days without certification	3
180 – 364 days	3
365 days and over	6

* Provided the AI Center Veterinarian can certify that the bull has not been housed with female cattle since reaching the age of 30 days.

Note: international bovine semen export regulations vary among countries and not all CSS testing procedures for Bovine Campylobacteriosis and Bovine Venereal Trichomoniasis are accepted for export.

5. Bovine Viral Diarrhea Virus (BVDV): All bulls and mount animals entering CSS approved AI centers must be tested for viremia 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 21 days after entry into the isolation facility. Furthermore, all bulls are to be evaluated by a testing program to detect persistent testicular infection.

   a. Diagnostic Test: The animal must be subjected to a PCR test on whole blood **OR** a virus isolation test on whole blood with a minimum of two (2) passages performed in bovine cell culture with a negative result as demonstrated by testing the cell culture by PCR or by staining of the cell culture by immunofluorescence (FA) or immunoperoxidase (IP) methods.

b. Diagnostic Specimens: PCR or virus isolation test on whole blood.

c. Demonstration of persistent BVDV infection: If BVDV is demonstrated by PCR **OR** by virus isolation followed by PCR, FA, or IP in cell culture on whole blood, the animal is to be isolated from other cattle and retested not less than 21 days by PCR on whole blood **OR** virus isolation test on whole blood with a minimum of two (2) passages performed in bovine cell culture with a negative result as demonstrated by testing the cell culture by PCR or by staining of the cell culture by immunofluorescence (FA) or immunoperoxidase (IP) methods. Demonstration of BVDV a second time is considered indicative of persistent infection and the animal is not eligible to enter the resident herd of the CSS approved AI center and must be removed from the isolation facility immediately.

d. Demonstration 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 tests conducted at least 14 days apart using PCR tests on whole blood **OR** virus isolation tests on whole blood with a minimum of two (2) passages performed in bovine cell culture with a negative result as demonstrated by testing the cell culture by PCR or by staining of the cell culture by immunofluorescence (FA) or immunoperoxidase (IP) methods.

   Bulls from which BVDV has been isolated but are later proven to be free of persistent infection must have samples of any semen collected and processed within the 30 days preceding and 30 days following the date of positive PCR or virus isolation test on whole blood, subjected to a PCR test from each collection code. A negative result is required for a collection code in order for it to be released for distribution. Any collection code with a positive result must be destroyed and not subject to further distribution.

e. If an animal demonstrates the presence of BVDV, after moving the animal apart from other cattle, the remaining cattle in the isolation facility will require a retest at least 14 days after separation of the positive animal using a PCR test on whole blood **OR** a virus isolation test on whole blood with a minimum of two (2) passages performed in bovine cell culture with a negative result as demonstrated by testing the cell culture by PCR or by staining of the cell culture by immunofluorescence (FA) or immunoperoxidase (IP) methods.

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:

a. Test bulls during the isolation period for BVDV by the serum neutralization (SN) test **OR** antibody ELISA test for both types I and II on serum. All bulls that test positive must have one negative PCR test on a sample of fully extended frozen semen. Frozen semen samples are to be transported to the diagnostic lab at cryogenic temperatures [i.e., liquid nitrogen or liquid nitrogen vapor tank].

-OR-

b. The bulls must have one (1) negative PCR test on a sample of fully extended frozen semen. Frozen semen samples are to be transported to the diagnostic lab at cryogenic temperatures [i.e., liquid nitrogen or liquid nitrogen vapor tank].

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

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

Any bull that has a positive PCR test or virus isolation test on whole blood during isolation and demonstrated NOT to be persistently infected, must have negative PCR tests on samples of fully extended frozen semen collected on at least three (3) separate occasions at intervals of not less than seven (7) days. These semen samples for testing for must be collected at least 21 days after the last positive PCR test or virus isolation test on whole blood.

Note: international bovine semen export regulations vary among countries and not all CSS testing procedures for BVDV are accepted for export.

All bulls or mount animals in the isolation facility shall be maintained in continuous isolation from all other livestock species and other bovine animals that are not of equivalent health status. If an individual bull or mount animal from the isolation facility is permitted contact with bovine animals that are not of equivalent status, he shall be removed immediately from the isolation facility and shall not be permitted re-entry until such time as he has completed another cycle of pre-entry and the tests prescribed, therefore.

RESIDENT HERD

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

The following tests shall be conducted for all bulls and mount animals at six (6) month intervals:

1. Bovine Tuberculosis: official intradermal Caudal Fold Test (CFT); the result shall be negative.

   Should the animal have a CFT result other than negative, a confirmatory test with negative result for bovine tuberculosis in accordance with USDA regulations and guidelines is required.

   Note: international bovine semen export regulations vary among countries and not all USDA approved bovine tuberculosis tests are accepted for export.

2. Bovine Brucellosis: One buffered brucella antigen test (Card or BAPA) **OR** one complement fixation (CF) test **OR** one fluorescent polarization assay (FPA) test on serum with a negative result.

   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 federal or state veterinary officials.

3. Bovine Leptospirosis: A microscopic agglutination test (MAT) on serum for serotypes *L. pomona*, *L. hardjo*, *L. canicola*, *L. icterohaemorrhagiae*, and *L. grippotyphosa* with a negative result.

   A negative result is preferred for each serotype. However, if the result is not negative (that is positive at 1:100 or 1:200) for any serotype, the bovine must have at least one retest for that serotype conducted at least 14 days following the previous test. Cattle that are **negative at 1:400** on two consecutive tests have a stabilized low titer and considered negative.

4. Bovine Campylobacteriosis and Bovine Venereal Trichomoniasis:

    a. Microscopic examination of cultured preputial material **OR** PCR test of preputial material collected from the fornix of the prepuce for *Tritrichomonas foetus*, with a negative result.

    b. Examination of cultured preputial material **OR** PCR test of preputial material collected from the fornix of the prepuce for *Campylobacter fetus* subsp. *venerealis*, with a negative result.

All bulls or mount animals in the resident herd shall be maintained in continuous isolation from all other livestock species and other bovine animals that are not of equivalent health status. If an individual bull or mount animal from the resident tested herd is permitted contact with any bovine animals that are not of equivalent status, 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 pre-entry and isolation and the tests prescribed therefore, except as provided for in the next paragraph below.

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 collection 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 (formerly *Haemophilus somnus*)
    Campylobacter fetus subsp. *venerealis*

2. Effective microbiological control is the condition in which the number of specific pathogenic microbial organisms potentially present are reduced to below the threshold of infectivity or destroyed.

3. An acceptable protocol is the in vitro exposure 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 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 Appendix 1.

REFERENCES

1. Shin SJ, Lein DH, Patten VH, and Ruhnke HL. A new antibiotic combination for frozen bovine semen. 1. Control of mycoplasmas, ureaplasmas, *Campylobacter fetus* subsp. *venerealis* and *Haemophilus somnus*. Theriogenology. 1988; 29:577-591.

2. Lorton SP, Sullivan JJ, Bean B, Kaproth M, Kellgren H, and Marshall C. A new antibiotic combination for frozen bovine semen. 2. Evaluation of seminal quality. Theriogenology;1988; 29:593-607.

3. Lorton SP, Sullivan JJ, Bean B, Kaproth M, Kellgren H, and Marshall C. A new antibiotic combination for frozen bovine semen. 3. Evaluation of fertility. Theriogenology. 1988; 29:609-614.

4. Shin SJ, Kim SG. Comparative efficacy study of bovine semen extension: 1-step vs 2-step procedure. 18th Technical Conference on Artificial Insemination and Reproduction. National Association of Animal Breeders, Columbia, MO, September 29-30, 2000, Proceedings, p. 60-62.

* * * *

CERTIFIED SEMEN SERVICES
8413 Excelsior Drive, Suite 140
Madison, WI 53717
Phone: +1 608 827 0277
Fax: +1 608 827 1535
Website: www.naab-css.org

APPENDIX 1

GTLS ANTIBIOTIC AND EXTENDER PROCEDURES AND CONDITIONS

ANTIBIOTICS

1. Neat Semen Treatment

a. Each ml of neat semen will be treated with 0.02 ml of the antibiotic combination (GTLS) containing the following active ingredients.[1]

- gentamicin 500 µg
- tylosin 100 µg
- lincomycin 300 µg
- spectinomycin 600 µg

b. The addition of these antibiotics should allow a ***minimum*** of three minutes for the antibiotics to be in contact with the neat semen before the addition of any extender.[1]
c. These procedures are required regardless of extender type used.[1,4]

2. Premixed Powdered Antibiotics

a. Powdered premixed GTLS antibiotics are formulated to contain the following concentrations of ***active*** antibiotics per 0.02 ml of final solution:

- gentamicin 500 µg
- tylosin 100 µg
- lincomycin 300 µg
- spectinomycin 600 µg

b. Premixed powdered GTLS antibiotics must be stored according to manufacturer's specifications until reconstitution.
c. Reconstitution of antibiotics must be completed using purified water (sterile double distilled, deionized, or reverse osmosis.) and mixed according to manufacturer's instructions.
d. Reconstituted premixed powdered antibiotics:

- must be used on the day of mixing and not held over
  -or-
- may be divided into aliquots and stored at -20°C, or colder, for up to 7 days. Aliquots must be thawed at room temperature and only used on the day of thawing and not refrozen.[5]

e. Extenders must be used on the day the reconstituted antibiotics are added.

3. General GTLS Antibiotic Requirements

a. Specific antibiotics required must be USP, EP or equivalent grade:

- gentamicin sulfate powder CAS number 1405-41-0 (≥590 µg/mg)[6]
- tylosin tartrate powder CAS number 74610-55-2 (≥900 µg/mg)[6]
- lincomycin hydrochloride powder CAS number 859-18-7 (≥790 µg/mg)[6]
- spectinomycin sulfate tetrahydrade powder CAS number 64058-48-6 (650-850 µg/mg)[6]
  -or-
  spectinomycin hydrochloride powder CAS number 22189-32 (≥603 µg/mg)[6]

b. Each antibiotic lot must have a detailed Certificate of Analysis available with the following information:

- Batch/lot number
- Expiration date/date of manufacture
- Assay for Potency or active ingredient or activity or mg/ml or µg/mg
- Purity or % inert ingredients or impurity %
- Composition or component % for gentamicin and tylosin

c. All of the antibiotic concentrations expressed herein are for ***active*** units of antibiotic. Potency values will vary between batches. However, by meeting the minimum required µg/mg for each antibiotic in section 3.a., antibiotics can be measured by actual weight instead of needing to calculate weigh based on potency.
d. Certificates of Analysis, for each antibiotic type and lot, must be made available upon request.
e. Antibiotics manufactured for industry use or formulated for in-house use must meet these criteria.
f. **Note**: Antibiotics obtained from some sources may contain deleterious materials that could affect antibiotic efficacy or sperm quality. Requesting a detailed Certificate of Analysis, as described in Section 3.b., will assure a minimum level of testing, with potency values and will list inert ingredients that could be problematic. Please contact Certified Semen Services for guidance.

EXTENDERS

4. 2-Step Extender

a. Neat semen treatment of 0.02 ml of GTLS/ml neat semen, with a ***minimum*** of 3 minutes contact time. See Section 1.
b. Non-glycerol fraction will be prepared to contain the following concentrations of active antibiotics before being added to semen:[1]

- gentamicin 500 µg per ml
- tylosin 100 µg per ml
- lincomycin 300 µg per ml
- spectinomycin 600 µg per ml

c. The non-glycerol extender 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, while cooling to 5°C. Then the glycerol containing extender can be added.[1]
d. The glycerol fraction of extender may contain no more than 10 percent of the antibiotic concentration listed under Section 4.b. non-glycerol fraction of extender. This addition cannot be counted towards the final concentration of antibiotics listed in Section 4.b.
e. The glycerol fraction of extender should be added to the non-glycerol fraction of extender plus semen at a 1 to 1 ratio (Glycerol fraction volume = Non-glycerol volume + semen volume).

f. The above procedures should yield the following final active concentration of GTLS /ml of frozen semen.[1]

- gentamicin 250 µg per ml
- tylosin 50 µg per ml
- lincomycin 150 µg per ml
- spectinomycin 300 µg per ml

g. Extender with antibiotics must be used the day of mixing with excesses discarded.

5. 1-Step Extender

a. Neat semen treatment of 0.02 ml of GTLS/ml neat semen, with a ***minimum*** of 3 minutes contact time. See Section 1.
b. 1-step extender will be prepared to contain the following concentrations of active antibiotics before being added to semen:[4]

- gentamicin 500 µg per ml
- tylosin 100 µg per ml
- lincomycin 300 µg per ml
- spectinomycin 600 µg per ml

c. The above procedures, for 1-step extender will yield the following final concentration of GTLS /ml of frozen semen.[4]

- gentamicin 500 µg per ml
- tylosin 100 µg per ml
- lincomycin 300 µg per ml
- spectinomycin 600 µg per ml

Note: The final concentration of antibiotics is doubled that of the 2-step extender protocol.

d. All semen should be held in contact with the 1-step extender for a ***minimum*** of two hours, while cooling to 5°C.
e. Extender with antibiotics must be used the day of mixing with excesses discarded.

PROCEDURES AND DEVIATIONS

6. Required Processing Procedures

a. Extender must be approved by CSS. See Appendix 1A
b. Antibiotic treatment to neat semen and extenders must be followed per Sections 1., 4., and 5. of this Appendix 1.
c. GTLS antibiotic/neat semen contact time must be at least three minutes.
d. Semen in non-glycerol extender fraction must cool for at least two hours to 5°C before the glycerol fraction is added.
e. Semen in one-step extender must cool for at least two hours to 5°C before additional processing steps.
f. Other procedures described in Sections 1 through 5 of this Appendix 1.

7. Deviation from Required Processing Procedures

It has been shown that extender composition and processing procedures may affect the efficacy of GTLS microbial control.[1] Therefore, if there is deviation from any of the required procedures listed in this Appendix 1, CSS evaluation and possible antibiotic efficacy testing will be necessary.

a. A written request for an exception will be made to the Service Director(s) of CSS.
b. When any new extender composition is presented for evaluation, and possible efficacy testing, the components and proportions must be shared with CSS and will be held in strict confidence.
c. The CSS Service Director(s) will determine whether the deviation will require testing for antibiotic efficacy.
d. If needed, appropriate efficacy testing will be done at a laboratory approved by CSS that has demonstrated competency for carrying out these analyses.
e. The test results will be returned from the laboratory to the CSS Service Director and the requesting organization.
f. If the results demonstrate efficacy equal to or greater than obtained by Shin[1] then approval to use the procedure will be granted by CSS.
g. All fees and expenses for evaluation of the deviation and or efficacy testing will be paid by the organization making the request and will be billed through CSS.

REFERENCES:

1. Shin SJ, Lein DH, Patten VH, and Ruhnke HL. A new antibiotic combination for frozen bovine semen. Control of mycoplasmas, ureaplasmas, *Campylobacter fetus* subsp. *venerealis* and *Haemophilus somnus*. Theriogenology. 1988; 29:577-591.

2. Lorton SP, Sullivan JJ, Bean B, Kaproth M, Kellgren H, and Marshall C. A new antibiotic combination for frozen bovine semen. 2. Evaluation of seminal quality. Theriogenology;1988; 29:593-607.

3. Lorton SP, Sullivan JJ, Bean B, Kaproth M, Kellgren H, and Marshall C. A new antibiotic combination for frozen bovine semen. 3. Evaluation of fertility. Theriogenology. 1988; 29:609-614.

4. Shin SJ, Kim SG. Comparative efficacy study of bovine semen extension: 1-step vs 2-step procedure. 18th Technical Conference on Artificial Insemination and Reproduction. National Association of Animal Breeders, Columbia, MO, September 29-30, 2000, Proceedings, p. 60-62.

5. Althouse GA. University of Pennsylvania New Bolton Center. A study of GTLS antibiotic stability at various temperatures and times. March 2021. NAAB/CSS sponsored research project.

6. U.S. Pharmacopeia reference monographs by CAS number.

APPENDIX 1A

CSS APPROVED EXTENDERS

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

Andromed-CSS 1-step* – Minitube USA	**Optidyl CSS** 1-step* – IMV Int'l Corp dba IMV Technologies
Andromed-CSS 2-step – Minitube USA	**OptiXcell** 1 step* – IMV Int'l Corp dba IMV Technologies
Biladyl 2-step - Minitube USA	**Steridyl-CSS** 1-step* – Minitube USA
BioXcell 1-step* - IMV Int'l Corp dba IMV Technologies	**Steridyl-CSS** 2-step - Minitube USA
BioXcell 2-step - IMV Int'l Corp dba IMV Technologies	**Triladyl-CSS** 1-step* – Minitube USA
BoviPro Cryoguard 1-step* – Minitube of America, Inc.	**Viam Pac** 2-step - Viam Pac Inc.
BoviPro Cryoguard 2-step - Minitube of America, Inc.	**BoviFree CSS** 1-step* – Minitube USA
Concentrated Semen Ext. 1-step* - Continental Plastic Corp.	**BoviFree CSS** 2-step – Minitube USA
Concentrated Semen Ext. 2-step - Continental Plastic Corp.	**GameteGuard® -FB 1-Step Extender** *– Membrane Protective Technologies, Inc.
Bovine -Ext™ One Part 1-step* - Reproduction Provisions	**GameteGuard® -FB 2-Step Extender** – Membrane Protective Technologies, Inc.
Bovine -Ext™ Two Part 2-step - Reproduction Provisions	

* 1-step extenders require additional quantities of GTLS antibiotics as described in Appendix 1Section 3. CSS does not endorse the inclusion of antibiotics in liquefied extender concentrates from the manufacturer and those antibiotics should not be considered toward the final required concentrations.

2. The following five extenders have been tested for antibiotic efficacy for control of microbial organisms.[1, 4] Use of the current GTLS antibiotic combination in extenders 1, 2 and 3 did not adversely affect post-thaw motility.[2] Additionally, extenders 1 and 3 were evaluated for fertility.[3] Extenders 4, and 5 were not evaluated for semen quality or fertility. The final composition of each extender is as follows:

Extender	Extender Composition	Approved for
1. 20% Egg Yolk Citrate	20% Egg yolk 2.12 gm % sodium citrate dihydrate 0.183 gm % citric acid monohydrate 7.0% glycerol	2-step[1]
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	1-step[4] (this protocol was not evaluated for semen quality or fertility) 2-step[1]
3. Heated Whole Milk	7.0% glycerol	2-step[1]
4. Plus-X	Plus-X, as supplied by distributor. 7.0% glycerol	2-step[1]
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	2-step[1]

3. To determine if an extender, not listed here, has been approved contact CSS.
4. In addition to the extenders listed here, other extenders have been approved by CSS through procedures outlined in Appendix 1 Section 7. These are proprietary extenders used by individual organizations and are held in strict confidence with CSS.

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 (January 21, 2021):

	TESTING ENVIRONMENTS		
	Pre-entry To Isolation (Within 30 days prior to entering the CSS Isolation facility)	**Isolation** (Testing before entry into a resident herd and semen release)	**Resident Herd** (Semen collection center)
Physical Examination	Conducted by Category II USDA accredited veterinarian.		
Bovine Tuberculosis	Negative intradermal Caudal Fold Test **or** official confirmatory test. (Within 60 days prior to entry)	Negative intradermal Caudal Fold Test **or** official confirmatory test at least 60 days after pre-entry test and 21 days after entering the CSS Isolation facility.	Negative intradermal Caudal Fold Test **or** official confirmatory test at 6-month intervals.
Bovine Brucellosis	Negative official test of state where bull is located (if required for movement). Negative CF **or** Card **or** BAPA **or** FPA test on serum for entry into the CSS Isolation facility.	Negative CF **or** Card **or** BAPA **or** FPA test on serum at least 30 days after pre-entry testing and 21 days after entering the CSS Isolation facility.	Negative CF **or** Card **or** BAPA **or** FPA test on serum at 6-month intervals.
Bovine Viral Diarrhea Virus	Negative PCR **or** antigen capture ELISA **or** virus isolation test performed on either whole blood or serum.	Negative PCR **or** virus isolation (2 passages minimum) test performed on whole blood at least 21 days after entry into the CSS Isolation facility **AND** Negative PCR test of processed frozen semen before release for use **OR** Negative antibody ELISA **or** SN test for BVDV types I and II on serum.	
Leptospirosis	Negative **or** stabilized low titer MAT test on serum for 5 serotypes important in the US.[1]	Negative **or** stabilized low titer MAT test on serum for 5 serotypes important in the US[1] no sooner than 30 after	Negative **or** stabilized low titer MAT test on serum for 5 serotypes important in the US[1] at 6-month intervals.

	TESTING ENVIRONMENTS		
	Pre-entry To Isolation (Within 30 days prior to entering the CSS Isolation facility)	**Isolation** (Testing before entry into a resident herd and semen release)	**Resident Herd** (Semen collection center)
		pre-entry test and 21 days after entering isolation.	
Campylobacteriosis		Series of negative PCR **or** culture tests of preputial material completed at least at weekly intervals: • Bulls under 180 days of age[2] with certification[3] - negative on 1 test. • Bulls under 365 days of age[2] - negative on 3 tests. • Bulls 365 days of age or older[2] - negative on 6 tests.	Negative PCR **or** culture test of preputial material at 6-month intervals.
Trichomoniasis		Series of negative PCR **or** microscopic examinations of cultured preputial material tests completed at least at weekly intervals: • Bulls under 180 days of age[2] with certification[3] - negative on 1 test. • Bulls under 365 days of age[2] - negative on 3 tests. • Bulls 365 days of age or older[2] - negative on 6 tests.	Negative PCR **or** microscopic examination of cultured preputial material test at 6-month intervals.

[1] *L. pomona*, *L. hardjo*, *L. canicola*, *L. icterohaemorrhagiae*, *L. grippotyphosa*

[2] Age on the day of entry into the CSS Isolation facility.

[3] Providing AI Center Veterinarian can certify that bull has not been housed with female cattle since reaching the age of 30 days.

APPENDIX 3

EXAMPLE: CSS ISOLATION TESTING CHECKLIST

Following is an **EXAMPLE** checklist and schedule of health testing to be completed during the CSS Isolation testing interval:

PRE-ENTRY TO ISOLATION TESTING:

☐ Bulls intended to enter a CSS approved AI Center shall be:

1. Healthy
2. Free of infectious or contagious disease
3. Not under quarantine

   AND
4. Should not be used for natural service after physical exam and testing
5. Should be isolated from other cattle after physical exam and testing

☐ Tests and exam must be performed, and results known before bull may enter the CSS Isolation facility

1. Within 60 days of Isolation Entry:
   - ☐ **Bovine Tuberculosis** - Negative intradermal Caudal Fold Test **or** official confirmatory test
2. Within 30 days of Isolation Entry:
   - ☐ **Physical Examination** - Performed by Category II USDA Accredited Veterinarian or equivalent
   - ☐ **Bovine Brucellosis** - Negative BAPA **or** Card **or** CF **or** FPA on **serum**
     (Note and comply with interstate transportation regulations)
   - ☐ **Bovine Leptospirosis** - Negative or low stable titer MAT on **serum**
     5 serovars (*L. pomona*, *L. hardjo*, *L. canicola*, *L. icterohaemorrhagiae*, *L. grippotyphosa)*
   - ☐ **BVD** - Negative PCR **or** antigen capture ELISA **or** Virus Isolation on **whole blood or serum**

ISOLATION TESTING

☐ Sampling may not commence until Pre-Entry test results are known and after the bull has entered the CSS Isolation facility.

*Note - Testing for Trichomoniasis and Campylobacteriosis completed at least at weekly intervals

1. Week 1 (Day 0):
   - ☐ **Trichomoniasis** - Negative culture **or** PCR of preputial material [1]
   - ☐ **Campylobacteriosis** - Negative culture **or** PCR of preputial material [1]
2. Week 2 (Day 7):
   - ☐ **Trichomoniasis** - Negative culture **or** PCR of preputial material [1, 2, 3]
   - ☐ **Campylobacteriosis** - Negative culture **or** PCR of preputial material [1, 2, 3]
3. Week 3 (Day 14):
   - ☐ **Trichomoniasis** - Negative culture **or** PCR of preputial material [1, 2]
   - ☐ **Campylobacteriosis** - Negative culture **or** PCR of preputial material [1, 2]

4. Week 4 (Day 21):
   - ☐ **Trichomoniasis** - Negative culture **or** PCR of preputial material [1, 2]
   - ☐ **Campylobacteriosis** - Negative culture **or** PCR of preputial material [1, 2]
5. Week 5 (Day 28):
   - ☐ **Trichomoniasis** - Negative culture **or** PCR of preputial material [1]
   - ☐ **Campylobacteriosis** - Negative culture **or** PCR of preputial material [1]
6. Week 6 (Day 35):
   - ☐ **Trichomoniasis** - Negative culture **or** PCR of preputial material [1]
   - ☐ **Campylobacteriosis** - Negative culture **or** PCR of preputial material [1]
   - ☐ **Bovine Brucellosis** - Negative BAPA **or** Card **or** CF **or** FPA on **serum** [4, 5]
   - ☐ **Bovine Leptospirosis** - Negative or low stable titer MAT on **serum** [4, 5]
     5 serovars (*L. pomona*, *L. hardjo*, *L. canicola*, *L. icterohaemorrhagiae*, *L. grippotyphosa*)
   - ☐ **BVD** - Negative PCR **or** Virus Isolation (2 passages) on **whole blood** [4, 5]
   - ☐ **BVD** - Negative SN **or** antibody ELISA (Types I and II) on serum **OR** Negative PCR on processed frozen semen [6]
7. Week 9 (Day 63):
   - ☐ **Bovine Tuberculosis** - Negative intradermal Caudal Fold Test **or** official confirmatory test [4, 7]

[1] If bull is equal to or greater than 365 days of age on the day of entry into CSS Isolation
[2] If bull is less than 365 days of age on the day of entry into CSS Isolation
[3] If bull is less than 180 days on the day of entry into CSS Isolation with veterinary certification of same sex housing since 30 days of age
[4] Conducted at least 21 days after entering CSS Isolation
[5] Conducted at least 30 days after the pre-entry test
[6] Conducted at any time during the isolation period or later if the bull is not of semen producing age but prior to semen dispatch.
[7] Conducted at least 60 days after the pre-entry test