

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 ($\geq 590 \mu\text{g}/\text{mg}$)⁶
 - tylosin tartrate powder CAS number 74610-55-2 ($\geq 900 \mu\text{g}/\text{mg}$)⁶
 - lincomycin hydrochloride powder CAS number 859-18-7 ($\geq 790 \mu\text{g}/\text{mg}$)⁶
 - spectinomycin sulfate tetrahydrate powder CAS number 64058-48-6 ($650\text{-}850 \mu\text{g}/\text{mg}$)⁶
 - or-
 - spectinomycin hydrochloride powder CAS number 22189-32 ($\geq 603 \mu\text{g}/\text{mg}$)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 $\mu\text{g}/\text{mg}$
 - 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 $\mu\text{g}/\text{mg}$ for each antibiotic in section 3.a., antibiotics can be measured by actual weight instead of needing to calculate weight 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 Shin¹ 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:

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