



## Laboratory Guidebook Notice of Change

Chapter new, **revised**, or archived: MLG 34.03

Title: Bioassay for the Detection, Identification and Quantitation of Antimicrobial Residues in Meat and Poultry Tissue

Effective Date: 05/25/11

### Description and purpose of change(s):

This MLG chapter has been revised to incorporate the following updated material:

- Numbered all tables throughout the chapter and updated information in Tables 1, 2 and 5
- Removed Section 34.4A Appendix and incorporated information into MLG chapter.
- Removed information in Section 34.7.1.2 (Critical Steps in Plate Preparation) and incorporated it into Section 34.7.1.1
- Section 34.7.1.2.d.ii (Bioassay Plate Quality Control) emphasizes the zone sizes of the plates 1, 2, 4, 5, and 7 shall be within 10% of the historical average of the standard reference concentration.
- Section 34.8.1 states for a new standard curve, the average zone size of the new standard reference concentration shall be within 10% of the historical value of the previous standard reference concentration.
- Updated Section 34.8.2 providing overall guidelines for making all antibiotic working standards including the following:
  - Stated that QC requirements for preparing an antibiotic standard solution before using it to test samples.
  - Changed Section 34.8.2.1 from tetracycline (antibiotic working standards) to general guidelines; Section 34.8.2.1.1 (Preparation of tetracycline and oxytetracycline solutions) was incorporated into the new Section 34.8.2.1
  - Removed Sections 34.8.2.1.2, and 34.8.2.2 to 34.8.2.7 which included preparation of chlortetracycline, penicillin and ampicillin, streptomycin and dihydrostreptomycin, erythromycin, tylosin, neomycin and gentamicin, respectively.
  - Sections 34.8.2.2 and 34.8.2.3 were changed to include information on Specific Antibiotic Guidelines and Summary of Antibiotic Reference Standards (was Section 34.14), respectively.

**The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use.**

- Under Section 34.8.3 Standard Curves, provided clarity in Section 34.8.3.2 Calculations by explaining it for use of software and manually. These sections were titled 34.8.3.2 and 34.8.3.3, Software Calculations of a Standard Curve and Manual Calculation of a Standard Curve, respectively.
- Information in Section 34.7.1.2 was incorporated into Section 34.7.1.1 providing general instructions and critical control points for bioassay quality control plates.
- Changed the order of sections for ease of understanding the method. Instructions for reading the bioassay (Section 34.11) was placed in front of instructions for anticipated results for each individual antibiotic assay (Section 34.10).
- Since the summary of antibiotic reference standards (Section 34.14) was incorporated into Section 34.8.2 antibiotic working standards, the sections regarding additional testing procedures and references were changed to Sections 34.14 and 34.15, respectively.

Additional changes include:

- Minor formatting and wording changes
- Added Section 34.1.3 which provided information on the validation of new or revised assays.
- The introduction concerning the preparations of bacterial cultures was removed in Section 34.6
- The Table in Section 34.9.1 was removed because an example of how to set up a sample bag with the appropriate pH buffer was not needed. This information can be obtained from Table 6.

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## **34.1 Introduction**

### **34.1.1 General**

Antibiotics are used in food animals for the prevention and treatment of infectious diseases and for growth promotion. When antibiotics are used properly, they should not leave detectable residues in edible tissues above levels allowed by Federal regulations. Inappropriate use of antibiotics is undesirable for two main reasons. Residues may produce toxic or allergic reactions in susceptible individuals who eat meat or poultry that contains antibiotic residues; and microorganisms may develop resistance to frequently used antibiotics. Because of the importance of detecting antibiotics in food animals, the United States Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS) developed a Bioassay System, a seven plate agar diffusion assay using monolayer plates, which can detect and quantitate a range of antibiotic residues found in meat and poultry products including kidney, liver, and muscle.

### **34.1.2 Minimum Limits of Applicability**

The lowest concentration of a known reference standard that produces a zone of inhibition at least 8 mm on a particular bioassay plate is referred to as the minimum inhibitory concentration (MIC). The MIC of each bioassay antibiotic defines the limits of detection for that antibiotic residue.

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### **34.1.3 Validation of New or Revised Assays**

In order to validate a new assay or changes to an assay, or to detect the presence of a new antibiotic in tissue, it is necessary to analyze tissues that have been artificially inoculated with the target antibiotic.

## **34.2 Safety Precautions**

The microorganisms used in the bioassay are Biosafety Level 1 and 2 organisms. Safe laboratory practices should be followed in working with all organisms. Exercise caution in working with hazardous chemicals by wearing appropriate protective safety clothing, gloves, and eyeglasses or face shields. Consult a Material Safety Data Sheet (MSDS) for each hazardous chemical before working with it. Collect hazardous chemical wastes in separate containers and dispose of them in accordance with the standard chemical waste management procedures for your laboratory. FSIS personnel should follow the FSIS Laboratory Environmental, Health, and Safety Handbook.

## **34.3 Quality Control (QC) Practices**

All glassware must be chemically clean. Stainless steel bioassay plates or cylinders (spiders) must be carefully cleaned so that no organic, antibiotic, or chemical residues remain. All media used shall be prepared and tested in accordance with the quality control practices described in the FSIS Quality System documents which includes the Microbiology Laboratory Guidebook Appendix I Media and Reagents, the FSIS Lab-Wide SOPs, and the individual laboratory's Work Instructions. Additional quality control practices and critical control points (CCP) are described in this chapter under the appropriate sections.

## **34.4 Equipment, Reagents, and Media**

### **34.4.1 Equipment and Supplies**

- a. Stainless steel cylinders or bioassay plates with 6 wells spaced at 60° intervals (stainless steel bioassay plates are referred to in this chapter as 'spiders').
- b. Plastic Petri dishes, 100 x 15 mm, high quality with flat bottoms
- c. Timer
- d. Stomacher® Model 80, model 400, or equivalent equipment
- e. Water bath, 45°C - 55°C range, capable of maintaining constant temperature  $\pm 2^\circ\text{C}$
- f. Zone reading device capable of reading in millimeters, such as a Fisher- Lilly antibiotic zone reader or calipers

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- g. Sterile forceps and test tubes
- h. Incubators set at  $29 \pm 1^{\circ}\text{C}$ ,  $35 \pm 1^{\circ}\text{C}$ , and  $37 \pm 1^{\circ}\text{C}$
- i. Forceps for stainless steel bioassay spiders
- j. Vacuum drying oven, capable of maintaining a constant temperature in the range of  $60 \pm 4^{\circ}\text{C}$  to  $110 \pm 4^{\circ}\text{C}$  and vacuum of 6 mm atmospheric pressure or as needed for antibiotic standard preparation.
- k. Sterilizing oven (dry-heat, sterilizing ovens must be capable of maintaining a temperature of  $160 \pm 10^{\circ}\text{C}$  for at least 180 to 240 minutes, depending on capacity.)
- l. Desiccator
- m. Micropipettors and suitable tips
- n. Class A volumetric flasks; 10, 25, 50, 100, and 200 ml, or equivalent
- o. Media bottles, 250-300 ml and other sizes as needed, or equivalent
- p. Bags for stomaching samples (Whirl-Pak bags, Tekmar filter bags or equivalent) (Optional)
- q. Class A graduated, volumetric pipettes; assorted sizes
- r. Refrigerator ( $2-8^{\circ}\text{C}$ )
- s. Freezer ( $-10^{\circ}\text{C}$  or lower)
- t. Balance, analytical
- u. Balance, 0.1 to 200 gram capacity
- v. Colony counter
- w. Blender, Tissuemizer™ or similar
- x. Vortex mixer
- y. Positive displacement pipettor, Hamilton syringe, or similar device for dispensing small volumes of viscous solutions
- z. Semi-logarithmic graph paper or computer with spreadsheet program
- aa. Magnetic stirrer (optional)

#### **34.4.2 Reagents**

Recipes are given in the MLG Appendix 1, Media and Reagents except as noted.

- a. 0.1 M phosphate buffer,  $\text{pH } 4.5 \pm 0.1$
- b. 0.1 M phosphate buffer,  $\text{pH } 6.0 \pm 0.1$
- c. 0.1 M phosphate buffer,  $\text{pH } 8.0 \pm 0.1$
- d. 0.2 M phosphate buffer,  $\text{pH } 8.0 \pm 0.1$
- e. Butterfield's Phosphate Buffer,  $\text{pH } 7.2$
- f. Reference antibiotics as needed - Minimum: Tetracycline hydrochloride, penicillin G potassium salt, streptomycin sulfate, erythromycin, neomycin sulfate

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- g. 0.1 N HCl
- h. 0.1 N NaOH
- i. 1.0 N HCl
- j. 1.0 N NaOH
- k. 0.85% NaCl solution (sterile saline)
- l. Penicillinase Concentrate, equivalent to at least 10,000,000 Kersey Units/ml
- m. Specific antibiotics as required for quantitation, USP or equivalent
- n. Antibiotic sensitivity disks: penicillin P2 or P10, tetracycline Te30, streptomycin S10, erythromycin E15, and neomycin N5 (e.g. BD Sensi-Discs: BD Biosciences, 7 Loveton Circle, Sparks, MD 21152 or equivalent product)
- o. Isotonic saline with 0.01% Tween as described in Section 34.6.1.2

#### **34.4.3 Media**

Media Recipes are given in the MLG Appendix 1, Media and Reagents.

- a. Antibiotic Medium 4 (or Antibiotic Medium 2 with dextrose)
- b. Antibiotic Medium 5
- c. Antibiotic Medium 8
- d. Antibiotic Medium 11
- e. Plate Count Agar
- f. AOAC approved biochemical identification systems such as API Staph Trac<sup>®</sup>, API strips<sup>®</sup> or VITEK<sup>®</sup> (bioMérieux, Inc., Durham, NC 27717), MicroID<sup>®</sup> (Organon Teknika Corp., Durham, NC); **or** biochemical reagents as needed to identify the microorganisms per SOP LW-0015 **or** Certificate of analysis for cultures.

#### **34.5 Test Organisms**

##### **34.5.1 Microorganisms Used for the Bioassay System**

The following test organisms are used in the preparation of agar plates used for the standard curves and the seven-plate bioassay system. Use aseptic technique when working with all bacterial cultures.

- a. *Kocuria rhizophila* or *K. rhizophila* (formerly *Micrococcus luteus*), ATCC 9341a (KR) (see Tang *et al.*)
- b. *Kocuria rhizophila* (*K. rhizophila*), ATCC 15957 (KRER)
- c. *Staphylococcus epidermidis* (*S. epidermidis*), ATCC 12228 (SE)



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- d. *Bacillus cereus* var. *mycoides* spores (*B. cereus*), ATCC 11778 (BC)
- e. *Bacillus subtilis* spores (*B. subtilis*), ATCC 6633 (BS)

Preparations of all cultures are available commercially. The vegetative cultures above are available as: LYFO DISK lyophilized organisms from MicroBioLogics, Inc., 217 Osseo Avenue North, St. Cloud, MN 56303-4452: *K. rhizophila* ATCC 9341a, *K. rhizophila* ATCC 15957 and *S. epidermidis* ATCC 12228. Spore suspensions of *B. subtilis* ATCC 6633 and *B. cereus* ATCC 11778 are maintained in 50% ethanol. Other suppliers may be used if equivalent preparations are available.

#### **34.5.2 Confirmation of Properties of Test Organisms**

Culture identity and purity must be verified. A certificate of analysis verifying identity and purity is acceptable for new lots of bacterial cultures.

The antibiotic sensitivity patterns of the test organisms must be confirmed. Prepare bioassay plates listed in Table 1 as described in Section 34.7 Preparation of Bioassay Plates, **with the exception that penicillinase should not be added to the agar**. Use three plates of specified medium for each organism tested. Each plate will be tested with all five of the antibiotics listed in the table, plus the standard reference for that specific plate. Place a spider or six cylinders on each plate. Add 200 microliters of each antibiotic concentration specified in the table below to individual wells. Add 200 microliters of the standard reference to a well. Antibiotics with a '≥' should not produce a zone of inhibition greater than 8 mm at the indicated concentration on that plate. For the other antibiotic-plate combinations, a measurable zone of inhibition of 8 to 15 mm should be produced at the concentrations listed. Replace any test organism that is not consistently capable of meeting these criteria with a new ATCC culture.

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**Table 1. Antibiotic Sensitivity Patterns of Cultures**

Test Organism	Medium No.	Antibiotic concentration in µg per mL				
		Erythromycin	Neomycin	Penicillin	Streptomycin	Tetracycline
<i>K. rhizophila</i> 9341a	2	0.05	5.0	0.0125	≥ 200	0.5
<i>K. rhizophila</i> 9341a	11	0.025	1.5	0.0125	≥200	0.4
<i>K. rhizophila</i> 15957	11	≥ 200	1.5	0.0125	1.0	0.2
<i>S. epidermidis</i> 12228	11	0.2	0.3	5	≥200	250
<i>B. cereus</i> 11778	8	0.25	2.00	20.00	1.00	0.08
<i>B. subtilis</i> 6633	5	0.04	0.25	0.0125	0.2	0.08

### 34.6 Use of Bacterial Culture Preparations

#### 34.6.1 Use of Commercial Lyophilized Pellets Containing Vegetative Cells

Individual pellets for all three lyophilized test organisms (*K. rhizophila* ATCC 9341a, *K. rhizophila* ATCC 15957, and *S. epidermidis* ATCC 12228) are commercially available. The use of lyophilized pellets reduces the following: the potential for the sudden loss of a viable and well characterized culture; the potential for the loss of desired properties such as antibiotic sensitivity and resistance patterns; and labor-intensive procedures for producing vegetative cell cultures. Store the lyophilized pellets at 2–8°C.

##### 34.6.1.1 Materials Required

- a. LYFO DISK lyophilized pellets: *K. rhizophila* ATCC 9341a, *K. rhizophila* ATCC 15957 and *S. epidermidis* ATCC 12228.
- b. Isotonic saline with 0.01% Tween
- c. Sterile forceps and test tubes
- d. Positive displacement pipette, Hamilton syringe or equivalent

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**34.6.1.2 Rehydration of Assay Organisms from Pellets**

- a. Prepare isotonic saline with 0.01% Tween as described below. Quantities may be varied if the proportions are retained. An appropriate measuring device (such as a positive displacement pipette, Hamilton syringe or equivalent product) is needed to dispense the Tween, as the solution is very viscous.
  - i. Dissolve 8.5 g of sodium chloride in 1000 mL of distilled water.
  - ii. Add 0.1 mL of Tween 80.
  - iii. Allow ample time for the solution to stir to insure thorough mixing.
  - iv. Autoclave at 121°C for 15 minutes @ 15 lbs of pressure or filter sterilize.
- b. Rehydrate the pellet. Aseptically transfer the lyophilized pellet(s) into a sterile tube and add  $2.0 \pm 0.08$  mL of room temperature saline-Tween solution for each pellet. Let suspension stand for approximately 90 minutes.
- c. Vortex the pellet mixture to aid in the dispersion of the pellet.

**Note:** If the pellet does not dissolve, it may be necessary to briefly warm the suspension to about 40°C. The pellet suspension may be stored at 2 – 8°C for up to 5 days.

**34.6.2 Spore Suspensions of *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633**

This protocol uses *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633 spore suspensions in ethanol. Store spore suspensions for each culture at 2–8°C.

**34.6.2.1 Preparation of Working Spore Suspensions**

To determine the number of spores/mL in each new spore stock suspension, perform plate counts following the procedure described in Section 34.6.3 Determination of Number of Colony Forming Units (cfu). Incubate at  $35 \pm 1^\circ\text{C}$  for 46 to 50 hours.

To determine the dilution required to obtain  $1 \times 10^6$  spores/mL in 50% ethyl alcohol use the following formula:

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$$D = W/S$$

Where:

S = Stock concentration; W = Working concentration; D = Dilution

Example:

Stock concentration =  $5 \times 10^8$

Working concentration desired =  $1 \times 10^6$

$$D = 1 \times 10^6 / 5 \times 10^8$$

$$D = 1/500$$

### 34.6.3 Determination of Number of Colony Forming Units (cfu)

To determine the number of bacteria per mL of each culture suspension, prepare a tenfold dilution series ( $10^{-2}$  to  $10^{-8}$ ) in the following manner.

- a. Pipette 1 mL of well mixed (use vortex mixer) stock suspension into 99 mL of Butterfield's phosphate buffer for the  $10^{-2}$  dilution. Use a separate, sterile 10 mL pipette to prepare each dilution. Pipette 10 mL of the  $10^{-2}$  dilution into a 90 mL dilution blank for the  $10^{-3}$  dilution. Continue to prepare additional dilutions by adding 10 mL from the newly prepared dilution to the next 90 mL dilution blank. Make sure that each dilution is mixed thoroughly before using it to make the next dilution. Repeat as needed to finish the dilution series.
- b. Pipette 1 mL of each dilution into two 100 x 15 mm petri dishes, and add 15 mL of molten Plate Count Agar which has been cooled in a water bath to  $48 \pm 2^\circ\text{C}$ . Mix by swirling or tilting the plates to disperse the inoculum evenly throughout the medium. Incubate for 24 to 48 h at  $35 \pm 1^\circ\text{C}$  until colonies are easily visible. Some cultures grow more rapidly than others. Avoid incubating so long that colonies merge.
- c. Count duplicate plates in a suitable range (30 – 300 colonies) on a colony counter. Record the dilution and number of colonies found. Average the counts obtained and record the counts per mL for each dilution of plates. Note: If counts are obtained outside the countable range on the  $10^{-8}$  plate, the procedure must be repeated using higher dilutions.

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- d. Using the counts obtained, calculate and record the stock concentration. The vegetative cell stocks should contain at least  $1 \times 10^8$  cfu/mL. Preparations with lower counts of vegetative cells may be used, but will require greater quantities to prepare the plates. The minimum recommended count is  $5 \times 10^6$  cfu/ml. The spore suspensions should contain at least  $1 \times 10^6$  cfu/mL.

#### **34.6.4 Optimization of Bioassay Plate Bacterial Lawn**

Each antibiotic has a set of dilutions that are recommended for producing the standard curve. The standard curve is used for calculating the quantity of antibiotic residue in a sample. The lowest concentration of each standard curve dilution should produce a clear zone of inhibition on a bacterial lawn of the proper organism with the appropriate cfu/mL concentration. If necessary, adjust the bacterial lawn target level to optimize the bioassay results. To determine the amount of the rehydrated pellet or spore suspension necessary for making bioassay plates, use the following formula:

$$X = (TV)/C$$

Where:

X = amount of stock culture suspension to be added to the molten agar (mL)

T = target (desired) number of bacterial cells in the lawn (cfu/mL)

V = volume of molten agar (mL)

C = concentration of stock culture suspension (cfu/mL)

**EXAMPLE 1:**

T =  $4 \times 10^4$  cfu/mL lawn

C =  $8 \times 10^6$  cfu/mL *B. cereus* stock culture suspension

V = 150 mL agar

$$X = (TV)/C$$

$$X = \frac{(4 \times 10^4 \text{ cfu/mL})(150 \text{ mL})}{8 \times 10^6 \text{ cfu/mL}}$$

$$X = 6 \times 10^6 \text{ cfu} / 8 \times 10^6 \text{ cfu/mL}$$

$$X = 0.75 \text{ mL}$$

0.75 mL of *B. cereus* stock culture suspension should be added to the molten agar

**EXAMPLE 2:**

T =  $1 \times 10^5$  cfu/mL lawn

C =  $2 \times 10^8$  cfu/mL culture from pellet, in isotonic Tween solution

V = 100 mL agar

$$X = (TV)/C$$

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$$X = \frac{(1 \times 10^5 \text{ cfu/mL})(100 \text{ mL})}{2 \times 10^8 \text{ cfu/mL}}$$

$$X = 1 \times 10^7 \text{ cfu} / 2 \times 10^8 \text{ cfu/mL}$$

$$X = 0.05 \text{ mL}$$

0.05 mL of rehydrated lyophilized culture should be added to the molten agar

### 34.7 Preparation of Bioassay Plates

#### 34.7.1 General Instructions and Plate Quality Control

##### 34.7.1.1 General Instructions and Critical Control Points (CCP)

- a. To prepare each type of plate described below, prepare the required amount of the specified medium and temper it to  $48 \pm 2^\circ\text{C}$  in a water bath. If previously prepared agar is used, the agar should be heated gently to melt it. Do not melt prepared agar more than once. **Note:** Do not use a microwave oven to melt the agar.
- b. Organism pellets are rehydrated using a saline-Tween solution. Follow the procedure for preparation of plates located in the “bioassay plate summary table” below.
- c. Add the required volume of organism suspension to produce the desired concentration of organisms. See the examples in section 34.6.4 above.
- d. Add penicillinase if required for a specific plate type.
- e. Thoroughly mix the inoculated medium by swirling and/or using a pipette to draw the solution up and down several times. To make the plates, dispense 8.0 – 8.5 mL of the preparation into plastic petri plates (100 x 15 mm). (CCP)
- f. Quickly and gently swirl the plates so that the seeded agar uniformly covers the surface of the plate. Work rapidly to assure that the agar does not prematurely harden while pouring the plates. To assure uniform thickness, let the agar harden on a flat, level surface ensuring no holes or air bubbles (CCP).

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- g. Properly identify each bioassay plate. Mark a vertical line on the side of the bottom of the plate for proper alignment of stainless steel spider. The date of preparation or expiration, and identity of the preparer should also be recorded.
- h. Store the prepared plates at 2 – 8°C.
- i. Prepared plates must not be allowed to freeze or dry out.
- j. Plates may be used for up to 7 days after preparation.

**Table 2. Bioassay Plate Summary**

Plate No.	Antibiotic Media	Organism	Culture Preparation	Water Bath Incubation*	Penicillinase
1	No. 8	<i>B. cereus</i> ATCC 11778	None	45 min	1mL/100mL agar
2	No. 4	<i>K. rhizophila</i> ATCC 9341a	Rehydrate (90 minutes)	none	none
3	No. 4	<i>K. rhizophila</i> ATCC 9341a	Rehydrate (90 minutes)	none	1mL/100mL agar
4	No. 5	<i>B. subtilis</i> ATCC 6633	None	75 min	1mL/100mL agar
5	No. 11	<i>K. rhizophila</i> ATCC 9341a	Rehydrate (90 minutes)	none	1mL/100mL agar
6	No. 11	<i>K. rhizophila</i> ATCC 15957	Rehydrate (90 minutes)	none	1mL/100mL agar
7	No. 11	<i>S. epidermidis</i> ATCC 12228	Rehydrate (90 minutes)	none	1mL/100mL agar

\* Using the 48 ± 2°C water bath, incubate spores in the molten agar before adding penicillinase

**34.7.1.2 Bioassay Plate Quality Control**

- a. The inoculum concentrations specified for the preparation of each plate (Section 34.7.2 Preparation of Specific Plates) have been standardized to achieve a confluent cell lawn growth. The lowest antibiotic standard concentration to which the organism is sensitive should produce a significant, readable zone of inhibition. If this is not achieved, prepare plates with varying concentrations of the bacterial suspensions to determine the amount of inoculum that produces the desired zone of inhibition for each assay. If the

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concentration of the organisms suspended in the agar is changed, prepare a new standard curve before using the plates for the bioassay.

- b. The prepared plates **must not** be used if stored for more than seven days after preparation. Each batch of prepared plates must be performance tested before use.
- c. When plates are used for screening samples and identifying residues the following quality control criteria must be met.

Produce antibiotic sensitivity patterns as described in the “Plate QC: Antibiotic Sensi-Disc Patterns Table” at the end of this section. Sensi-Discs concentrations other than those specified may be used to determine the pattern. Plates that do not perform as expected must be discarded and new plates produced to meet the requirements.

- d. Plates that will be used for quantitative analysis, including plates for standard curves, should also meet the following QC procedures.
  - i. A clear zone of inhibition (at least 8 mm) must be produced with the lowest concentration of the plate-specific antibiotic standard-curve dilution. **Note:** Plates 3 and 6 are confirmatory plates and will not produce a zone of inhibition with the specific antibiotic.
  - ii. Plates 1, 2, 4, 5, and 7 must also be tested with the plate-specific standard reference concentration. Zone sizes shall be within 10% of the historical average of the standard reference concentration.



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**Table 3. Plate QC and Antibiotic Sensi-Disc Patterns**

Plate No. & Organism	Agar	Antibiotic Sensi-Disc				
		Te30	P10	S10	E15	N5
1 - <i>B. cereus</i> ATCC 11778	# 8	S	R	S	S	S
2 - <i>K. rhizophila</i> ATCC 9341a	# 4 without penicillinase	S	S	R	S	R
3 - <i>K. rhizophila</i> ATCC 9341a	# 4 with penicillinase	S	R	R	S	R
4 - <i>B. subtilis</i> ATCC 6633	# 5	S	R	S	S	S
5 - <i>K. rhizophila</i> ATCC 9341a	#11	S	R	R	S	S
6 - <i>K. rhizophila</i> ATCC 15957	# 11	S	R	S	R	S
7 - <i>S. epidermidis</i> ATCC 12228	# 11	R	R	R	S	S

S = Zone of inhibition

R = No zone of inhibition

### 34.7.2 Preparation of Specific Plates

#### 34.7.2.1 Preparation of Plates with *B. cereus* and Penicillinase (BC, Plate 1)

- a. Pipette the required quantity of *B. cereus* (ATCC 11778) spores into melted, tempered Antibiotic Medium No. 8 to make a final concentration of  $5 \times 10^3$  cfu/mL of agar.
- b. Swirl the bottle gently to mix and incubate for 45 minutes in a  $48^\circ \pm 2^\circ\text{C}$  water bath.
- c. After incubation, add  $1 \pm 0.1$  mL of penicillinase per 100 mL of seeded medium (100,000 units per mL of agar).
- d. Mix the inoculated medium and prepare the plates as described in Section 34.7.1 General Instructions and Plate Quality Control.

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**34.7.2.2 Preparation of Plates with *K. rhizophila* (KR, Plate 2)**

- a. Pipette the required quantity of *K. rhizophila* cells (ATCC 9341a) into melted, tempered Antibiotic Medium No. 4 to make a final concentration of  $8 \times 10^5$  cfu/mL of agar.

**Note: No penicillinase is added to this agar preparation.**

- b. Mix the inoculated medium and prepare the plates as described in Section 34.7.1 General Instructions and Plate Quality Control.

**34.7.2.3 Preparation of Plates with *K. rhizophila* and Penicillinase (KR+P, Plate 3)**

- a. Pipette the required quantity of *K. rhizophila* cells (ATCC 9341a) into melted, tempered Antibiotic Medium No. 4 to make a final concentration of  $8 \times 10^5$  cfu/mL of agar.
- b. Add  $1 \pm 0.1$  mL of penicillinase per 100 ml of seeded media.
- c. Mix the inoculated medium and prepare the plates as described in Section 34.7.1 General Instructions and Plate Quality Control.

**34.7.2.4 Preparation of Plates with *B. subtilis* and Penicillinase (BS, Plate 4)**

- a. Pipette the required quantity of *B. subtilis* (ATCC 6633) spores into melted, tempered Antibiotic Medium No. 5 to make a final concentration of  $1 \times 10^4$  cfu/ml of agar.
- b. Swirl the bottle gently and **incubate for 75 minutes in a water bath at  $48 \pm 2^\circ\text{C}$ .**
- c. After incubation add  $1 \pm 0.1$  mL of penicillinase per 100 mL of seeded medium (100,000 units per mL of agar).
- d. Mix the inoculated medium and prepare the plates as described in Section 34.7.1 General Instructions and Plate Quality Control.

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**34.7.2.5 Preparation of Plates with *K. rhizophila* ATCC 9341a and Penicillinase (KR-11, Plate 5)**

- a. Pipette the required quantity of *K. rhizophila* cells (ATCC 9341a) into melted, tempered Antibiotic Medium No. 11 to make a final concentration of  $8 \times 10^5$  cfu/mL of agar.
- b. Add  $1 \pm 0.1$  mL of penicillinase per 100 mL of seeded media.
- c. Mix the inoculated medium and prepare the plates as described in Section 34.7.1 General Instructions and Plate Quality Control.

**34.7.2.6 Preparation of Plates with *K. rhizophila* ATCC 15957 and Penicillinase (KR-ER, Plate 6)**

- a. Pipette the required quantity of *K. rhizophila* cells (ATCC 15957) into melted, tempered Antibiotic Medium No. 11 to make a final concentration of  $2 \times 10^5$  cfu/mL of agar.
- b. Add  $1 \pm 0.1$  mL of penicillinase per 100 ml of seeded media (100,000 units/ml).
- c. Mix the inoculated medium and prepare plates as described in Section 34.7.1 General Instructions and Plate Quality Control.

**34.7.2.7 Preparation of Plates with *S. epidermidis* and Penicillinase (SE, Plate 7)**

- a. Pipette the required quantity of *S. epidermidis* (ATCC 12228) cells into melted, tempered Antibiotic Medium No. 11 to make a final concentration of  $1 \times 10^5$  cfu/mL of agar.
- b. Add  $1 \pm 0.1$  mL of penicillinase per 100 mL of seeded media.
- c. Mix the inoculated medium and prepare plates as described in Section 34.7.1 General Instructions and Plate Quality Control.

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### **34.8 Standard Curves**

Standard curves are generated for each antibiotic that is tested. The standard curve is calculated from the zones of inhibition produced by a series of antibiotic concentrations that have been tested on the plate type that is specific for that antibiotic. There are 5 antibiotic concentrations for each standard curve. The middle concentration is designated the antibiotic standard reference (SR) concentration. This standard reference concentration is also placed on plates along with unknown samples for quantitative analysis and is the point used for normalization between the quantitative analysis and the standard curve.

#### **34.8.1 Standard Curve QC Requirements**

Antibiotic standard curves should be prepared at a minimum of once every three months, or when a new lot of culture, reference standard, or plating media is used. The standard curve is expected to be relatively straight when plotted semi-logarithmically. For each new standard curve, the average zone size of the standard reference concentration (SR new) shall be within 10% of the historical value of the standard reference concentration (SR old). The previous standard curve should be compared to the new standard curve and trends, such as changes in slope, high and low points, and antibiotic zone sizes, should be tracked. If significant changes are observed an investigation may be warranted. If the lowest concentration of the standard curve (as defined in this method) does not produce a zone size of at least 8 mm in diameter, it may be necessary to change the antibiotic standard curve concentrations in your lab so that a zone size of at least 8 mm in diameter is produced at the lowest concentration. If the lowest concentration of the standard curve (as defined in this method) produces too large a zone, it may be necessary to change the antibiotic standard curve concentrations in your lab.

#### **34.8.2 Antibiotic Working Standards**

Authentic antimicrobial reference standards may be obtained from the United States Pharmacopeia, 12601 Twinbrook Parkway, Rockville MD 20852, and the Committee on National Formulary, American Pharmaceutical Association, 2215 Constitution Avenue, NW, Washington DC 20037. It is recommended that USP quantitation reference standards be used. If USP quantitation reference standards are not available, equivalent standards should be used.

It is necessary to dry several of the primary standards using a vacuum oven. Follow the manufacturer's instructions for drying, storing and preparing the antibiotic reference

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standards. Use clean Class A volumetric glassware. Stock solutions, 1000 parts per million (ppm<sup>1</sup>), should be prepared in an atmosphere of 50% relative humidity or less.

For quality control, compare data from new standards against data from the previous standards. Before any newly prepared antibiotic stock standard solution is used for testing samples it must pass the following QC requirements:

1. Zone size comparison - the average zone size of newly prepared standard reference concentration (SR new) must be within 10% of the historical value of the standard reference concentration (SR old).
2. MIC of new standard - the lowest concentration of the standard curve dilutions (MIC) must produce a zone size > 8mm.

#### **34.8.2.1 General Guidelines**

- a. Calculate the quantity of the specific antibiotic powder needed to produce 1000 ppm of free base in the desired volume. Use the free base content statement provided by the manufacturer (Example 1) or calculate the free base content by using the molecular formula information and purity information provided by the supplier. If the free base content is not available but the content is available in units, calculate the quantity by converting units to micrograms (Example 2). See the formula and examples below. Pure penicillin G potassium salt contains 1595 penicillin G units per milligram and this is equivalent to 88.8% penicillin G free base.

B = free base content (expressed decimally)

V = target volume

C = stock solution concentration in milligrams: 1000 µg/mL = 1mg/mL

X = quantity of antibiotic in micrograms

##### Example 1 – Calculating quantity using free base statement

B (free base content of the powder) = 88.8% (0.888)

V = 25 mL

C = 1 mg/mL

X = (C\*V)/B

X = 1(25)/0.888

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<sup>1</sup>Parts per million (ppm) is used in this chapter to express a quantity of 1 µg per mL.

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X = 28.15 mg.

Weigh 28.2 mg into a 25 mL volumetric flask and dilute to volume.  
(Analytical balances are capable of measuring to 4 decimal places in grams, i.e. to one tenth of a milligram. Therefore 28.15 mg is rounded to 0.0282 g)

Example 2 – Converting units to free base content before calculating quantity

The antibiotic powder contains 1490 units per milligram

Calculate the proportion:  $1490/1595 = B/0.888$

$B = (1490) (0.888)/1595$

$B = 0.8295$  (83.95%) penicillin G free base

$B = 0.8295$

$V = 25$  mL

$C = 1$  mg/mL

$X = 1(25)/0.8295 = 30.14$  mg

Weigh 30.1 mg into a 25 mL volumetric flask and bring to volume.

- b. Accurately weigh the required amount of antibiotic. Add the required volume of initial antibiotic solvent (as stated in Table 6, Standard Curve Summary) to produce a 1000 ppm ( $\mu\text{g/mL}$ ) stock solution. If the powder is transferred from the weighing container into another container for dilution, wash it into the container using the initial antibiotic solvent to ensure that the entire quantity is transferred.
- c. Store the stock solution at 2 – 8°C and use it within specified timeframe. See Table 4 Stock Solution Shelf Life.

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**Table 4. Stock Solution Shelf Life**

Antibiotic Standard	Stock Solution Expiration
Ampicillin	7 days
Chlortetracycline	7 days
Dihydrostreptomycin	7 days
Erythromycin	14 days
Gentamicin	30 days
Neomycin	30 days
Oxytetracycline	7 days
Penicillin	7 days
Streptomycin	7 days
Tetracycline	7 days
Tylosin	7 days

- d. Prepare the dilutions using appropriate phosphate buffer to obtain the specific concentrations listed in Table 6 Standard Curve Summary. Store dilutions at 2 – 8°C.

**34.8.2.2 Specific Antibiotic Guidelines**

a. Tetracycline

Tetracycline is light sensitive. All tetracycline powders and solutions should be protected from light. Amber glassware may be used for preparing solutions. If amber glassware is not used, the containers should be wrapped in foil or otherwise protected from light.

Oxytetracycline does not easily dissolve into solution. Two ways to facilitate the dispersion of the antibiotic into the solution is to: 1) use a magnetic stirrer and/or 2) maintain the antibiotic in the solution overnight at 2 – 8°C.

b. Erythromycin

A 1000 ppm stock solution of erythromycin can be prepared using two methods:

- i. Determine the quantity of erythromycin required to prepare a 1000 ppm solution. Weigh this amount into a suitable container (such as a clean disposable aluminum weigh boat or equivalent). Add about 2.0

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to 4.0 mL of pure methyl alcohol to the container and carefully swirl the container until the antibiotic powder is in solution. Add about 6 to 15 mL of buffer (pH 8.0, 0.2 M) to the methanol-antibiotic solution and mix again. Pour this solution into the appropriate volumetric flask. Rinse the weigh boat with additional buffer adding the rinse to the volumetric flask. Add buffer to the volumetric flask to bring it to volume.

- ii. Weigh antibiotic powder into a container. Calculate the amount of diluent needed to prepare 1000 ppm solution of the free base. Add about 2 to 3 mL of 30% methanol-buffer solution to dissolve the antibiotic powder. Subtract out the volume of methanol solution used. Add the remaining buffer solution to bring to the desired volume.

**NOTE:** For all other antibiotics, refer to Table 6.

**Table 5. Dilution Method for Preparing the Required Concentrations.**

From Solution*	Take (mL)	Dilute to (mL)	Resulting Concentration*
1000.0	5.0	50	100.00
100.0	4.0	50	8.0
8.0	4.0	25	1.28
8.0	2.0	25	0.64
8.0	1.0	25	0.32
8.0	1.0	50	0.16
8.0	1.0	100	0.08

\* In ppm (µg/ml)



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### 34.8.2.3 Summary of Antibiotic Reference Standards

In order to assist in the antibiotic bioassay, a summary table is presented below, describing appropriate antibiotic plates, microorganisms, buffers, and antibiotic standard concentrations necessary to prepare standard curves. A standard curve prepared by using these specific concentrations listed in the table below may be used to quantitate the amount of an antimicrobial residue in a tissue. For some antibiotics, the FDA NADA (New Animal Drug Application) method must be used for quantitation for regulatory purposes.

In the table below, the first concentration shown for a standard curve (column four) is used for demonstrating cell lawn sensitivity. The concentration shown in bold (middle dilution) is the SR concentration.

**Table 6. Standard Curve Summary**

Antimicrobial	Bioassay Plate	Antibiotic Initial Solvent	Antibiotic Buffer Diluent	Concentrations (µg/ml) used for the Standard Curve
Ampicillin	Plate 2, KR	0.1 M, pH 6.0	0.1 M, pH 6.0	0.01, 0.02, <b>0.04</b> , 0.08, 0.16
Chlortetracycline	Plate 1, BC	0.1 M, pH 4.5	0.1 M, pH 4.5	0.01, 0.02, <b>0.04</b> , 0.08, 0.16
Dihydrostreptomycin	Plate 4, BS	Sterile DI Water	0.1 M pH 8.0	0.15, 0.3, <b>0.6</b> , 1.2, 2.4
Erythromycin	Plate 5, KR-11	Methanol (see XXX)	0.2 M, pH 8.0	0.05, 0.1, <b>0.2</b> , 0.4, 0.8
Gentamicin	Plate 7, SE	0.1 M, pH 8.0	0.1 M, pH 8.0	0.1, 0.2, <b>0.4</b> , 0.8, 1.6
Neomycin	Plate 7, SE	0.1 M, pH 8.0	0.1 M, pH 8.0	0.25, 0.5, <b>1.0</b> , 2.0, 4.0
Oxytetracycline	Plate 1, BC	0.01 N HCl	0.1 M, pH 4.5	0.08, 0.16, <b>0.32</b> , 0.64, 1.28
Penicillin	Plate 2, KR	0.1 M, pH 6.0	0.1 M, pH 6.0	0.01, 0.02, <b>0.04</b> , 0.08, 0.16
Streptomycin	Plate 4, BS	Sterile DI Water	0.1 M, pH 8.0	0.1, 0.2, <b>0.4</b> , 0.8, 1.6
Tetracycline	Plate 1, BC	0.01N HCl	0.1 M, pH 4.5	0.08, 0.16, <b>0.32</b> , 0.64, 1.28
Tylosin	Plate 5, KR-11	0.2 M, pH 8.0	0.2 M, pH 8.0	0.2, 0.4, <b>0.8</b> , 1.6, 3.2

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### **34.8.3 Standard Curves**

#### **34.8.3.1 Procedure**

- a. Label the plates appropriately with antibiotic concentrations. Use triplicate plates for each concentration required for the standard curve, except for the standard reference (SR) concentration, for a total of 12 plates. Mark a vertical line on the side of the bottom of the plate for proper alignment of the stainless steel spider.
- b. Place sterile spiders or 6 sterile cylinders (evenly spaced around the perimeter of the plate) on each plate. Align the spider orientation hole with the line previously marked on the plate. The well below the orientation hole designates the starting point. Use care with spider forceps when placing metal spiders on the agar. Spiders may tear the agar if carelessly dropped or placed. Do not lift and re-place spiders on a plate.
- c. Fill three alternate wells with the antibiotic SR concentration and the other three wells with one of the four other concentrations of the antibiotic standard. Use  $200 \pm 4\mu\text{L}$  of solution for each well.
- d. Incubate the plates 16 to 18 hours at the appropriate temperature(s) as described in Section 34.9.3.1 General Instructions.
- e. After the plates have been incubated, remove the spiders or cylinders by inverting the test plates over a bucket or tub. Cover the spiders or cylinders with distilled water. Spiders and cylinders must be decontaminated chemically or by autoclaving. Spiders must be carefully cleaned so that no organic, antibiotic, or chemical residues remain.
- f. Read the diameters of the zones of inhibition to the nearest tenth of a millimeter using the zone reader or caliper. Start reading from the first well (marked line). Record the diameter of each zone of inhibition for both antibiotic concentrations on each plate. Also make the appropriate entry if no zone is produced. Record the data in an appropriate format such that the averages and corrected zones can be calculated.

#### **34.8.3.2 Software Calculations of a Standard Curve**

- a. Calculate the corrected standard curve and linear regression.

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- b. Apply a correction factor to compensate for between-plate variations. Each antibiotic dilution value (except for the SR) will be calculated from a set of 3 plates.
- c. Calculate cumulative average zones for all SR concentrations on the entire set of 12 plates.
- d. For each set of three plates:
  - i. Average the readings of the nine SR zones and the nine readings of the other concentration used for the standard curve.
  - ii. Subtract the nine-zone average of the SR from the cumulative SR average.
  - iii. Algebraically add the resulting value to the value of the average zone reading for the antibiotic concentration used on that set of plates.
- e. The standard curve is calculated on a semi-logarithmic graph (using the corrected values) with the zone diameters on the arithmetic scale and the antibiotic concentrations on the logarithmic scale. Calculate the linear regression from the corrected standard curve. Use the linear regression line for calculating values in quantitative analysis of test samples.

#### **34.8.3.3 Manual Calculation of a Standard Curve**

Average all 36 readings of the standard reference (SR) concentration from the 12 plates. This cumulative average is the correction point for the standard curve.

For each set of three plates:

- a. Average the readings of the nine SR zones and the nine readings of the other concentration used for the standard curve.
- b. Subtract the nine-zone average of the SR from the cumulative SR average.
- c. Algebraically add the resulting value to the value of the average zone reading for the antibiotic concentration used on that set of plates.

Example 1. If, in correcting the second concentration of the standard curve, the cumulative average of the 36 readings of the reference concentration is 20.0 mm and the average of the nine readings of the SR on this set of three plates is 19.8 mm, then the correction is + 0.2 mm. (20.0 minus 19.8 = 0.2). If the average of the

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second concentration on triplicate plates is 17.0 mm, then corrected value is 17.2 mm.

Example 2. If the grand average of the reference concentration is 20.0 mm but the average of the nine readings of the reference concentration on the set of plates is 20.3 mm, the correction factor would be '-0.3'. If the average of the second concentration on triplicate plates is 17.0 mm, then corrected value is 16.7 mm. (The sum of 17.0 and '-0.3' is 16.7.)

- d. Use the method described below to construct the curve.
- i. Plot the corrected values, including the correction point, on semi-logarithmic graph paper, using the log scale for the concentration (the concentration of antibiotic for the 'y' axis) and the arithmetic scale for the zone diameters (the adjusted value of a sample on the 'x' axis).
  - ii. Draw a line of best fit between the high and the low end points of a curve derived from the equations below.

$$L = (3 a + 2 b + c - e) / 5$$

$$H = (3 e + 2 d + c - a) / 5,$$

Where:

L and H = calculated zone diameters for the low and high concentrations, respectively, on the standard response line.

a, b, c, d, and e = the corrected average zone diameters for each concentration on the response line, where "a" equals the lowest concentration of antibiotic used and "e" equals the highest concentration used.

## **34.9 Bioassay Procedures for the Detection and Quantitation of Antibiotic Residues in Animal Tissues**

### **34.9.1 Sample Preparation and Storage**

Tissues suspected of containing antimicrobials should be handled so that freezing and thawing are kept to a minimum. Samples and sample extracts must be kept cold (see 34.5.1.d and e) at all times with allowances for brief excursions at room temperature during processing and testing. The laboratory should take measures to ensure good housekeeping.

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Environmental conditions should be controlled so that they do not adversely affect the quality of the test results. Clean and disinfect the work area with an appropriate disinfectant. Take care to assure disinfectant residue does not contaminate testing materials (i.e. stainless steel spiders and cutting utensils). Use a clean set of equipment for cutting, weighing, or blending each tissue. Keep tissues isolated from one another at all times to avoid commingling.

- a. Use Tekmar<sup>®</sup> filter bags, Whirl-Pak<sup>®</sup> bags, or equivalent. Label a bag with the sample identification, tissue type, and buffer pH. Four bags will be required for each tissue.
- b. Dice tissue into 0.5 cm pieces or homogenize a large enough portion of the sample to complete all anticipated tests. Use a blender (or similar apparatus) to homogenize the samples. The homogenized tissue portion should be frozen. If possible, retain an intact portion of the frozen tissue as a sample reserve.
- c. Weigh sample portions into the four, labeled bags. If a large amount of sample (i.e. > 100 g) is available, weigh  $10 \pm 0.2$  g of tissue (avoid fat) into each bag. If a small amount of sample (i.e. < 100g) is available, use  $5 \pm 0.1$  g per bag and make a note so that a proportional amount of diluent is added. Keep the sample to buffer ratio at 1 part sample to 4 parts buffer.
- d. For 10 gram samples, dispense  $40 \pm 1.0$  ml of the appropriate buffer into each bag. For 5 gram samples, use  $20 \pm 0.5$  ml of buffer. Stomach the diced muscle tissue samples for 60 seconds. Stomach the diced kidney and liver tissues for 30 seconds. Thoroughly mix samples into the buffer if the tissues have already been homogenized. After stomaching or mixing, allow the tissue to extract/settle for a minimum of 45 minutes before use. Follow the assay procedure as described below. The sample extracts should be refrigerated if they will be held for more than 2 hours before use.
- e. The extracts may be stored refrigerated for 24 hours, or frozen for 14 days for additional testing.

#### **34.9.2 Preparation of Spiked Tissues**

To prepare a daily process control sample, it is necessary to analyze tissues which have been artificially inoculated (spiked) with the antibiotic to be assayed. Follow the procedure described below:

- a. Select only those tissues from food animals that are known to be free of antimicrobials as defined by the MIC.

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- b. Prepare individual tissue samples and inoculate each with a known quantity of antibiotic. Individual samples may be inoculated with different types and quantities of antibiotic(s). Select concentration(s) that give a response that is expected to fall within the range of concentrations used for preparing the standard curve. Refer to Table 6 Standard Curve Summary.
- c. Follow the extraction procedures (Section 34.9.1 Sample Preparation and Storage) for these samples in parallel with non-inoculated (non-spiked) samples.
- d. Determine the recovery of the antibiotic from the spiked tissues following the procedures described for sample quantitative analysis (Section 34.9.3.4 Quantitative Analysis of Known Antibiotic Residues in Tissue). This information may be used in the laboratory's control charts.

### **34.9.3 Performing the Seven Plate Bioassay**

The bioassay can be performed in several combinations.

- a. A screening test may be done to determine the type of antimicrobial residue present, followed by the appropriate quantitative analysis (Section 34.9.3.2 Screening for Identification of Antimicrobial Residues).
- b. Alternatively, the full seven-plate bioassay may be done on the sample(s), accomplishing both identification and quantitation at the same time (34.9.3.3 Identification and Quantitative Analysis Using the Full 7-plate Bioassay).
- c. If the antimicrobial residue present is already known, the quantitative step may be performed to determine the concentration of the residue (34.10 Individual Antibiotic Assays).

Each of these 3 approaches is described below. The individual assays are described in Section 34.10 Individual Antibiotic Assays. Instructions on reading the plates are given in Section 34.11 Reading the Bioassay. Interpretation of results is found in Section 34.12 Additional Information on Test Interpretation.

**Note:** it is important to decontaminate spiders or cylinders after use. Chemical disinfection or sterilization by autoclaving may be used. In either case, it is essential that the spiders be carefully cleaned so that no organic, antibiotic, or chemical residues remain.

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### 34.9.3.1 General Instructions

These instructions are for use as a general guide to performing the bioassay.

- a. Obtain a sufficient number of plates and spiders for the assays that are to be performed. Label the plate lids according to the extracts under analysis. Mark a vertical line on the side of the bottom of the plate for proper alignment of the stainless steel spider.

The following plates are used with the extracts listed to test for each of the standard antibiotic residues. For the full assay, place the plates in the sequence listed.

Plate 1	BC	(tetracycline detection – pH 4.5 buffer extract)
Plate 2	KR	(beta-lactam detection – pH 6.0 buffer extract)
Plate 3	KR+P	(penicillin confirmation – pH 6.0 buffer extract)
Plate 4	BS	(streptomycin detection – pH 8.0, 0.1M buffer extract)
Plate 5	KR-11	(macrolide detection – pH 8.0, 0.2M buffer extract)
Plate 6	KRER	(erythromycin confirmation – pH 8.0, 0.2 M buffer extract)
Plate 7	SE	(aminoglycoside detection – pH 8.0, 0.1M buffer extract)

- b. To perform the assay:
  - i. Using a pair of spider forceps, GENTLY place one sterile spider on each plate. Align the spider orientation hole with the line previously marked on the plate to indicate the well serving as a starting point.
  - ii. Fill the wells with  $200 \pm 4 \mu\text{l}$  of the appropriate test sample extract or SR.
  - iii. Place the lids on the plates and incubate plates 1 through 6 at  $29 \pm 1^\circ\text{C}$  for 16 to 18 h. Incubate plate 7 at  $37 \pm 1^\circ\text{C}$  for 16-18 h.
  - iv. After the plates have been incubated, remove the spiders or cylinders by inverting the test plates over a bucket or tub. Cover the spiders or cylinders with distilled water. Decontaminate the spiders and cylinders chemically or by autoclaving. Carefully clean the spiders so that no organic, antibiotic, detergent, or chemical residues remain.

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**NOTE:** If an error is made in dispensing an extract, repeat the procedure with a fresh plate.

#### **34.9.3.2 Screening for Identification of Antimicrobial Residues**

- a. Arrange the full set of seven plates in the order described above (Section 34.9.3.1 General Instructions), and label them appropriately. Place a spider or a set of cylinders on each plate.
- b. Use the extracts prepared as described in Section 34.9.1 Sample Preparation and Storage. Fill one well on each of the seven plates with  $200 \pm 4 \mu\text{l}$  of the appropriate buffered sample extract. Repeat for additional samples. Make sure that a record is kept of the sample placement in the wells.
- c. Use the following antibiotic SRs in one well each day the screen test is run: Plate 1, tetracycline; Plates 2 and 3, penicillin; Plate 4, streptomycin; Plates 5 and 6, erythromycin; Plate 7, neomycin or gentamicin. Pipette  $200 \pm 4 \mu\text{l}$  of the SR concentration into the test well. When screen-testing samples, Sensi-Discs may be used in place of using reference dilutions on each plate.
- d. Incubate as described in Section 34.9.3.1 General Instructions.
- e. After incubation, remove the spiders or cylinders. Read and record the zones (or absence of zones) on each of the seven plates as described in 34.11 Reading the Bioassay.

#### **34.9.3.3 Identification and Quantitative Analysis Using the Full 7-plate Bioassay**

- a. For each tissue to be analyzed arrange the full set of seven plates in the order described above (Section 34.9.3.1 General Instructions), and label them appropriately.
- b. Place a spider or a set of 6 cylinders on each plate, aligning the spider orientation grove with the mark on the bottom edge of the plate.
- c. Moving clockwise from the starting well (which is marked), fill three alternate wells with  $200 \pm 4 \mu\text{l}$  of the appropriate buffered sample extract. Fill the other



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three with the appropriate SR antibiotic dilution. The entire plate will be used for each tissue. Refer to the list under Section 34.9.3.1 (General Instructions) to match the plates, extracts, and SR antibiotics. Include a plate with a blank tissue (negative) and a positive (spiked) tissue to indicate the degree of precision and accuracy of analysis. This is in addition to the SR controls that are used on each plate.

- d. Incubate as described in Section 34.9.3.1 General Instructions.
- e. After incubation, remove the spider or cylinders and read and record the zones (or absence of zones) on each of the seven plates as described in Section 34.11 "Reading the Bioassay."
- f. Use the appropriate standard curve to calculate the sample test results for quantitative determination.

**Note:** Some plates may identify multiple antibiotics. Further testing may be necessary to determine the specific antibiotic present. If the antibiotic identified differs from the standard reference antibiotic that was used in this assay proceed to Section 34.9.3.4 Quantitative Analysis of Known Antibiotic Residues in Tissue to quantitate the antibiotic.

#### **34.9.3.4 Quantitative Analysis of Known Antibiotic Residues in Tissue**

- a. To determine the amount of known antibiotic residue in the tissue extract use the plate and buffered extract specific for that residue.
- b. Use at least one plate for each individual muscle, kidney, and liver sample extract.
- c. Moving clockwise from the starting well (which is marked), fill three alternate wells with  $200 \pm 4 \mu\text{l}$  of the buffered sample extract. Fill the other three with the reference concentration of the known antibiotic standard solution. Include a plate with a blank tissue (negative) and a positive (spiked) tissue to indicate the degree of precision and accuracy of analysis. This is in addition to the SR controls that are used on each plate.
- d. Incubate as described in Section 34.9.3.1 General Instructions.

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- e. After incubation, read and record the zones (or absence of zones) on each of the seven plates as described in 34.11 Reading the Bioassay.
- f. Use the appropriate standard curve to calculate the sample test results for quantitative determination.

### **34.10 Reading the Bioassay**

- a. After the plates have been incubated, remove spiders or cylinders by inverting test plates over a bucket or similar receptacle. Cover the spiders or cylinders in the bucket with distilled water. Spiders and cylinders must be decontaminated chemically or by autoclaving (see Section 34.3 Quality Control (QC) Practices).
- b. Arrange test plates according to numerical sequence of the plates.
- c. Read plates starting clockwise from the first well (marked line) and read the zone of inhibition. A calipers or similar zone measuring device may be used. Record the diameter to the nearest tenth of a millimeter of each zone of inhibition for both the unknown and the standard reference antibiotic. Also, make the appropriate entry if no zone is produced.

### **34.11 Individual Antibiotic Assays**

The following sections describe the use of each of the individual bioassay plates. The 7 – plate bioassay was designed to identify a limited number of antibiotics that were in use at a particular point in time. The antibiotic industry and chemical methods have both evolved since the bioassay was developed. Now many of the antibiotics must be identified, confirmed, or quantitated using chemical methods. The initial screening role of the bioassay is to indicate the presence of a class of antibiotic.

#### **34.11.1 Tetracyclines – BC Plate 1**

This method is used in detecting the presence of all tetracyclines. This is followed by an identification procedure to determine which tetracycline compound is present.

##### **34.11.1.1 Performing the Assay**

- a. Use BC Plate 1 (Refer to Section 34.7.2.1).
- b. Add  $200 \pm 4 \mu\text{l}$  of the sample extracted in 0.1M, pH 4.5 buffer to the test wells.

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c. Tetracycline is the standard reference antibiotic for this plate.

d. Incubate the plates at  $29 \pm 1^\circ\text{C}$  for 16 to 18 hours.

**34.11.1.2 Tentative Identification of Tetracycline in Tissue from the Seven Plate Bioassay System**

The tetracyclines are tentatively identified by zones of inhibition (ZI)  $> 8$  mm on BC Plate 1. When the concentration of tetracycline is low, there may be no ZI on any other plate. Various concentrations of tetracyclines may produce zones on any or all of the plates except the Plate 7. The specific tetracycline compound may be identified using HPLC following the method in the USDA, FSIS Chemistry Laboratory Guidebook, Method # CLG-TET2 or other appropriately validated methods.

**34.11.1.3 Quantitation of Tetracycline in Tissue**

After the residue is identified, perform a quantitative analysis on BC Plate 1 using the identified antibiotic as the standard reference (SR). Use the appropriate standard curve to calculate the sample test results as described in Section 34.13 Calculating the Concentration of Antibiotic Residue in Tissue.

**34.11.2 Beta-Lactam Antibiotics– KR Plate 2 and KR + P Plate 3**

**34.11.2.1 Performing the Assay**

- a. Use KR Plate 2 (Refer to Section 34.7.2.2) and KR+P Plate 3 (Refer to Section 34.7.2.3).
- b. Add  $200 \pm 4$   $\mu\text{L}$  of the sample extracted in 0.1M, pH 6.0 buffer to the test wells.
- c. Penicillin G potassium salt is the reference antibiotic for these plates.
- d. Incubate the plates at  $29 \pm 1^\circ\text{C}$  for 16 to 18 hours.

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**34.11.2.2 Tentative Identification of Beta-Lactam Residues in Tissue from the Seven Plate Bioassay System**

The presence of beta-lactams like penicillin in a sample is indicated by  $ZI \geq 8$  mm on KR Plate 2 and no zone on KR+P Plate 3. The presence of ceftiofur (metabolite marker DCCD) in a sample is indicated by  $ZI > 8$  mm on KR Plate 2 and KR+P Plate 3. The identity of residues matching this plate pattern is confirmed by additional testing.

**34.11.2.3 Quantitation of Beta-Lactam Residues in Tissue**

Upon identification of the beta-lactam residue, perform quantitative analysis. Use the sample zone size, the Standard Reference (SR) zone size, and the standard curve of the identified antibiotic to calculate the sample test results for quantitative determination. Examples include penicillin ampicillin and amoxicillin. Refer to Table 6 Standard Curve Summary for additional antibiotics.

**34.11.3 Streptomycin and Dihydrostreptomycin – BS Plate 4**

**34.11.3.1 Performing the Assay**

- a. Use BS Plate 4 (Refer to Section 34.7.2.4).
- b. Add  $200 \pm 4$   $\mu$ L of the sample extracted in 0.1 M, pH 8.0 buffer to the test wells.
- c. Streptomycin is the reference antibiotic for this plate.
- d. Incubate the plates at  $29 \pm 1^\circ\text{C}$  for 16 to 18 hours.

**34.11.3.2 Tentative Identification of Streptomycin or Dihydrostreptomycin in Tissue from the Seven Plate Bioassay System**

The presence of streptomycin or dihydrostreptomycin is tentatively identified by  $ZI \geq 8$  mm on BS Plate 4. There will be no zones on any other plates if the residue is present at a low concentration. If it is in high concentration, there may be zones  $\geq 8$  mm on Plates 1 and 6. There should be no zones of inhibition on Plates 2, 3, 5, and 7. Identification must be confirmed by other methods. Spectinomycin and low

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concentrations of fluoroquinolones may produce similar patterns. The specific antibiotic must be confirmed by chemical tests.

#### **34.11.3.3 Quantitation of Streptomycin or Dihydrostreptomycin in Tissue**

Upon confirmation of streptomycin or dihydrostreptomycin, perform quantitative analysis using BS Plate 4. Use the sample zone size, the SR antibiotic zone size, and the appropriate standard curve to calculate the quantity of streptomycin in the sample as described in Section 34.13 Calculating the Concentration of Antibiotic Residue in Tissue.

#### **34.11.4 Erythromycin and Tylosin – KR-11 Plate 5 and KR-ER Plate 6**

##### **34.11.4.1 Performing the Assay**

- a. Use KR-11 Plate 5 (Refer to Section 34.7.2.5) and KR-ER Plate 6 (Refer to Section 34.7.2.6).
- b. Add  $200 \pm 4$   $\mu$ L of the sample extracted in 0.2 M, pH 8.0 buffer to the test wells.
- c. Erythromycin is the reference antibiotic for this assay.
- d. Incubate the plates at  $29 \pm 1^{\circ}$ C for 16 to 18 hours.

##### **34.11.4.2 Identification and Confirmation of Erythromycin and Tylosin in Tissue from the Seven Plate Bioassay System**

The presence of erythromycin in tissue is indicated by  $ZI > 8$  mm on KR-11 Plate 5 and no zones of inhibition on KR-ER Plate 6. Use the zone size on KR-11 Plate 5 and the appropriate standard curve to calculate the quantity of erythromycin residue present in the sample as described in Section 34.13 Calculating the Concentration of Antibiotic Residue in Tissue.

The presence of tylosin in tissue is indicated by  $ZI > 8$  mm on KR-11 Plate 5 and no zones of inhibition on KR-ER Plate 6. Use the zone size on KR-11 Plate 5 and the appropriate standard curve to calculate the quantity of tylosin residue present in

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the sample as described in Section 34.13 Calculating the Concentration of Antibiotic Residue in Tissue.

#### **34.11.4.3 Quantitation of Erythromycin and Tylosin in Tissue**

Upon confirmation of erythromycin, use the sample zone size on KR-11 Plate 5, the erythromycin SR zone size, and the appropriate standard curve to calculate the quantity of erythromycin in the sample. This plate is also used to calculate the quantity of tylosin residue in tissue using the tylosin SR dilution as described in Table 6 Standard Curve Summary.

#### **34.11.5 Neomycin and Gentamicin – SE Plate 7**

##### **34.11.5.1 Performing the Assay**

- a. Use SE Plate 7 (Refer to Section 34.7.2.7) prepared with antibiotic agar No. 11, *S. epidermidis* ATCC 12228 and penicillinase as described in Section 34.7.2.7).
- b. Add  $200 \pm 4$   $\mu$ l of the sample extracted in 0.1 M, pH 8.0 buffer to the test wells.
- c. Neomycin is the reference antibiotic for this assay.
- d. Incubate the plates at  $37 \pm 1$  °C for 16 to 18 hours.

##### **34.11.5.2 Identification of Neomycin and Gentamicin in Tissue from the Seven Plate Bioassay System**

Neomycin and gentamicin residues present in tissue at low concentrations ( $<1.0$   $\mu$ g/g) produces  $ZI > 8$  mm only on BS Plate 4 and SE Plate 7. The largest zones of inhibition are usually observed on SE Plate 7. At higher concentrations, neomycin and gentamicin may also produce zones of inhibition on additional plates. Macrolides and other aminoglycosides may produce similar patterns. Chemical analysis must be used to establish the identity of the antibiotic (Refer to Section 34.14.1 Chemical Methods of Antibiotic Residue Confirmation and/or Quantitation).

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### **34.11.5.3 Quantitation of Neomycin and Gentamicin in Tissue**

Use the sample zone size on SE Plate 7, the neomycin SR antibiotic zone size, and the appropriate standard curve to calculate the quantity of neomycin residue present in the sample as described in Section 34.13 Calculating the Concentration of Antibiotic Residue in Tissue. This plate is also used to calculate the quantity of gentamicin residue in tissue using the gentamicin SR dilution as described in Table 6 Standard Curve Summary in 34.8.2.1.3 Summary of Antibiotic Reference Standards.

### **34.12 Additional Information on Test Interpretation**

To identify an antibiotic, the pattern of the ZI produced by a sample should be compared with the patterns produced by known antibiotics. The presence and/or absence of ZI on each of the 7 plates and the relative sizes of the zones are used in interpreting the results. The pattern obtained may not match known patterns. In that case, alternative approaches may be taken. Further testing may be necessary using more dilute sample preparations, or using other methods of analysis. Some concentrations of antibiotic residues may produce patterns that differ from typical patterns for that antibiotic. Antibiotics, other than the ones described here, may produce similar or different patterns to the ones described under the individual antibiotic tests. Further discussion is found in Section 34.14 Additional Testing Procedures.

### **34.13 Calculating the Concentration of Antibiotic Residue in Tissue**

The zone sizes on the test plate are compared to the standard curve to calculate the residue concentration in the test sample. To compensate for day-to-day and plate-to-plate variations, the sample zone size must be adjusted so that it is comparable to the standard curve. To calculate the antibiotic concentration in a test sample, use the steps described below. Calculations may be done manually or with a validated computer program (i.e., appropriate statistical or mathematical program).

- a. Adjust the test sample zone size
  - i. Average the zone readings of the SR concentration and of the sample.
  - ii. Subtract the zone average for the SR on the test plate from the average standard curve SR concentration.
  - iii. Algebraically add the resulting value to the value of the average zone reading for the test sample.

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Example 1:

The average zone size of the SR concentration on the standard curve = 20.5 mm  
The average zone size of the SR concentration on the test plate = 19.1 mm  
The difference = 1.4 mm

The average zone size of the test sample = 17.3 mm.  
Add 1.4 mm to 17.3 mm = adjusted zone size of 18.7 mm. This is used to determine the concentration of the residue in the sample from the standard curve.

Example 2:

The average zone size of the SR concentration on the standard curve = 20.5 mm  
The average zone size of the SR concentration on the test plate = 21.1 mm  
The difference = -0.6

The average zone size of the test sample = 17.3 mm.  
Add '-0.6' to 17.3 mm = adjusted zone size of 16.7 mm. This is used to determine the concentration of the residue in the sample from the standard curve.

- b. Calculate the residue concentration using the adjusted sample zone size.
- i. With the adjusted value of the sample on the arithmetic scale for the zone diameters of the standard curve determine the concentration of antibiotic from the log scale for the antibiotic concentration. This may be done using a validated computer program.
  - ii. In calculating the final concentration in a tissue sample, take into consideration the dilution factor of the sample extract. This is usually five (one part of sample to 4 parts of buffer).
  - iii. In those instances where the sample zone size exceeds the highest concentration on the standard curve, dilutions of the extract may be made so that the test concentration falls within the linear range of the standard curve.
  - iv. Use the appropriate current standard curve in your calculations. Round off the calculated concentration to the nearest hundredth microgram.



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### 34.14 Additional Testing Procedures

#### 34.14.1 Chemical Methods of Antibiotic Residue Confirmation and/or Quantitation

The FSIS Chemistry Laboratory Guidebook describes chemical methods that may be used to identify and/or quantitate some residues identified in tissues. Additional chemical methods of identifying and quantitating antibiotic residues may also be available.

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