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Laboratory Guidebook
Notice of Change

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

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Description and purpose of change(s):

This Microbiology Laboratory Guidebook method chapter was revised to update the formatting and to meet the requirements of the laboratory's document control system and ISO 17025. Additional content includes a section on method detection limits, safety precautions, and previously approved changes. Sections that are not in current use have been moved to an appendix where they are available for reference or use in some circumstances. The general content has been re-written to clarify procedures and update the method.

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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.

The US Food and Drug Administration (FDA) antibiotic assay procedures have long been used to detect antibiotics in milk, other dairy products, animal feeds, and animal tissues[i]. These methods were developed to quantitate individual antibiotic residues. However, they did not identify the specific antibiotic residue present. Using the principles of these assays, the United States Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS) developed a Bioassay System which is a seven plate agar diffusion assay that can detect and quantitate a range of antibiotic residues found in meat and poultry products. Later, this assay system was further modified to replace bilayer plates with monolayer plates.

This Bioassay System utilizes bacteria that are relatively sensitive or resistant to a particular class of antibiotic. The bacteria are used in combination with specific antibiotic test agars and four pH-specific, buffered sample extracts. If a detectable antibiotic residue is present in a sample, it produces a zone of clearing (inhibition) on one or more of the test plates. Certain antibiotic residues can be identified according to their characteristic patterns of inhibition. A chart called an antibiogram was developed that depicts expected patterns of inhibition that specific antibiotics are expected to produce on the 7 plates. However, some patterns correspond to multiple antibiotic residues; these samples are then subjected to further testing. Once the antibiotic residue has been identified, the concentration of the residue present may be determined from the standard curve by comparing the zone sizes produced by the sample to that produced by a reference antibiotic standard of known concentration. The identity and concentration of certain antibiotics are determined using chemical tests after they are detected in the bioassay. Chemical methods are not covered in this chapter but are referenced in Section 34.15.2

34.1.2 Limits of Detection

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.

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 Practices (QC)

All glassware must be chemically clean. 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: 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 are described in this chapter under the appropriate sections.

34.4 Equipment, Reagents, and Media

34.4.1 Equipment and Supplies

a. Stainless steel bioassay plates or cylinders with 6 wells spaced at 60° intervals (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, 400, or equivalent
e. Water bath, 45 to 55°C range, capable of maintaining constant temperature ± 2°C
f. Zone reading device capable of reading in millimeters, such as a Fisher- Lilly antibiotic zone reader or calipers
g. Sterile forceps
h. Incubators set at 29 ± 1°C, 35 ± 1°C, and 37 ± 1°C
i. Forceps for stainless steel bioassay spiders
j. Vacuum drying oven, capable of maintaining a constant temperature in the range of 60 ± 4°C to 110 ± 4°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 ± 10°C for at least 180 to 240 minutes, depending on capacity.)
l. Vacuum desiccator
m. Micropipettors and suitable tips
n. Flasks, volumetric, 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. Pipettes, graduated, assorted sizes
r. Refrigerator (2-8°C)
s. Freezer (-10°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 devise 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, pH 4.5 $\pm$ 0.1
b. 0.1 M phosphate buffer, pH 6.0 $\pm$ 0.1
c. 0.1 M phosphate buffer, pH 8.0 $\pm$ 0.1
d. 0.2 M phosphate buffer, pH 8.0 $\pm$ 0.1
e. Butterfield's Phosphate Buffer, pH 7.2
f. Reference antibiotics as needed - Minimum: Tetracycline hydrochloride, penicillin G potassium salt, streptomycin sulfate, erythromycin, neomycin sulfate
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 (e.g. Difco Sensi-Discs: BD Biosciences, 7 Loveton Circle, Sparks, MD 21152): penicillin P2 or P10, tetracycline Te30, streptomycin S10, erythromycin E15, and neomycin N5
o. Isotonic saline with 0.01% Tween as described in Section 34.6.1.2
p. Dextrose for use in preparation of antibiotic agar No. 2

34.4.3 Media (Media Recipes are given in the MLG Appendix 1, Media and Reagents)

a. Antibiotic Medium 2, with 0.1% 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®, API strips® or VITEK® (bioMérieux, Inc., Durham, NC 27717), MicroID® (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. *Micrococcus luteus,* ATCC 9341a (MLA)
b. *Micrococcus luteus,* ATCC 15957 (MLER)
c. *Staphylococcus epidermidis,* ATCC 12228 (SE)
d. *Bacillus cereus* var. *mycoides* spores, ATCC 11778 (BC)
e. *Bacillus subtilis* spores, ATCC 6633 (BS)

The vegetative cultures above are available commercially as: LYFO-DISK lyophilized organisms from MicroBioLogics, Inc., 217 Osseo Avenue North, St. Cloud, MN 56303-4452: *M. luteus* ATCC 9341a, *M. luteus* ATCC 15957 and *S. epidermidis* ATCC 12228. Spore suspensions of *B. subtilis* ATCC 6633 and *B. cereus* ATCC 11778 in 50% ethanol are available from MedTox Diagnostics, Inc, Burlington, NC 27215. Other suppliers may be used if suitable 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. If a certificate of analysis is not

available, or if culture identity and purity must be confirmed, follow the procedures described in the Appendix to this chapter.

The antibiotic sensitivity patterns of the test organisms must be confirmed. Prepare bioassay plates listed in the table below as described in Section 34.7 **with the exception that penicillinase should not be added to the agar.** Place a 'spider' or 5 cylinders on each plate. Add 200 microliters of the antibiotic concentrations specified in the table below to individual wells. Each prepared plate will be tested with all four of the antibiotics listed in the table. 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.

Table 34.5.2 Antibiotic Sensitivity Patterns of Cultures

Test Organism	Medium No.	Antibiotic concentration in mg per ml				
		Erythromycin	Neomycin	Penicillin	Streptomycin	Tetracycline
M. luteus 9341a	2	0.05	5.0	0.0125	> 200	0.5
M. luteus 9341a	11	0.025	1.5	0.0125	250	0.4
M. luteus 15957	11	> 200	1.5	0.0125	1.0	0.2
S. epidermidis 12228	11	0.075	0.25	0.8	1.5	250
B. cereus 11778	8	0.25	2.00	20	1.00	0.08
B. subtilis 6633	5	0.04	0.25	0.0125	0.2	0.08

34.6 Use of Bacterial Culture Preparations

The following material assumes the use of the specified commercial bacterial preparations. Instructions for the preparation of fresh and preserved vegetative cell cultures are located in the appendix to this chapter. Those instructions may be followed as an alternative approach to the use of commercial preparations.

34.6.1 Use of Commercial Lyophilized Pellets Containing Vegetative Cells

Individual pellets for all three lyophilized test organisms (*M. luteus* ATCC 9341a, *M. luteus* ATCC 15957, and *S. epidermidis* ATCC 12228) are now available commercially. The use of lyophilized pellets reduces the potential for sudden loss of a viable, well characterized culture, reduces the potential for loss of desired properties such as antibiotic sensitivity and resistance patterns, and reduces 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 organisms (Micro-Bio-Logics, 217 Osseo Avenue North, St. Cloud, MN 56303-4452) *M. luteus* ATCC 9341a, *M. luteus* ATCC 15957 and *S. epidermidis* ATCC 12228.]
b. Isotonic saline with 0.01% Tween
c. Sterile forceps

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) is needed to dispense the Tween, as the solution is very viscous.

   - Dissolve 8.5 g of sodium chloride in 1000 ml of distilled water.
   - Add 0.1 ml of Tween 80.
   - Allow ample time for the solution to stir to insure thorough mixing.
   - 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 ± 0.08 ml of room temperature saline-Tween solution for each pellet. Let this stand for approximately 90 minutes. It may be necessary to 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 *Bacillus cereus* ATCC 11778 and *B. subtilis* ATCC 6633

This protocol uses commercially available *Bacillus cereus* ATCC 11778 and *B. subtilis* ATCC 6633 spore suspensions in ethanol from MedTox Diagnostics, Inc. Laboratory-prepared spore suspensions may also be used. See the appendix to this chapter for instructions on preparing spore suspensions. Store all spore suspensions at 2–8°C.

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. If high counts are obtained (outside the countable range on these plates) the procedure must be repeated using higher dilutions.

a. Pipette one 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 one 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 ± 2°C. See the Microbiology Laboratory Guidebook Media Appendix for directions. Mix by swirling or tilting the plates to disperse the inoculum evenly throughout the medium. Incubate for 24 to 48 h at 35 ± 1°C or 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.

d. Using the counts obtained, calculate and record the stock concentration. The vegetative cell stocks should contain at least 1 x 10^{8} cfu/ml. The spore suspensions should contain at least 1 x 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 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 x 10^4 cfu/ml lawn
C = 8 x 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 x 10^6 cfu/8 x 10^6 cfu/ml
X = 0.75 ml
0.75 ml of *B. cereus* stock culture suspension should be added to the molten agar

EXAMPLE 2:
T = 1 x 10^5 cfu/ml lawn
C = 2 x 10^8 cfu/ml culture from pellet, in isotonic Tween solution
V = 100 ml agar

X = (TV)/C
X = (1×10^5 cfu/ml)(100 ml)

2×10^8 cfu/ml
$X = 1 \times 10^7$ cfu/2×10^8 cfu/ml
X = 0.05ml
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

- To prepare each type of plate described below, prepare the required amount of the specified agar and temper it to 48 ± 2° 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.
- Add the required volume of cell suspension to produce the desired concentration of organisms. See the examples in section 34.6.4 above.
- Add penicillinase if required.
- Mix the cell-agar solution by swirling and/or pipetting it several times. To make the plates, dispense 8.0 to 8.5 ml of the preparation into plastic petri plates (100 x 15 mm).
- Tilt or swirl the plates **gently** so that the agar uniformly covers the surface of the plate. Let the agar harden on a flat, **level** surface.
- 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.
- Store the prepared plates at 2-8°C.
- Prepared plates must not be allowed to freeze or dry out
- Plates may be used for up to 5 days after preparation.

Bioassay Plate Summary Table

Plate No.	Antibiotic Agar	Organism	Culture Preparation	Water Bath Incubation*	Penicillinase
1	No. 8	*Bacillus cereus* ATCC 11778	None	45 min	1ml/100ml agar

2	No. 2 (with dextrose)	*Micrococcus luteus* ATCC 9341a	Rehydrate (90 minutes)	none	none
3	No. 2 (with dextrose)	*Micrococcus luteus* ATCC 9341a	Rehydrate (90 minutes)	none	1ml/100ml agar
4	No. 5	*Bacillus subtilis* ATCC 6633	None	75 min	1ml/100ml agar
5	No. 11	*Micrococcus luteus* ATCC 9341a	Rehydrate (90 minutes)	none	1ml/100ml agar
6	No. 11	*Micrococcus luteus* ATCC 15957	Rehydrate (90 minutes)	none	1ml/100ml agar
7	No. 11	*Staphylococcus epidermidis* 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 Critical Steps in Plate Preparation

- Assure thorough mixing of organisms and penicillinase throughout the agar before pouring the plates.
- Use no less than 8.0 ml and no more than 8.5 ml of seeded agar for each plate.
- Assure that the entire plate is covered with a uniform layer of seeded agar and that the agar layer is smooth with no holes or air bubbles.
- Work rapidly to assure that the agar does not prematurely harden while pouring the plates.
- Incubate the spore-inoculated agars for the required times before making plates.
- Pour the plates on a flat **level** surface to assure that when the agar hardens it has a uniform thickness.

34.7.1.3 Bioassay Plate Quality Control

The inoculum concentrations specified for the preparation of each plate (Section 37.7.2) 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

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

The prepared plates must not be used if stored for more than five days after preparation. Each batch of prepared plates must be performance tested before use. Plates must meet the following criteria.

- 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.
- Plates 1, 2, 4, 5, and 7 must also be tested with the plate-specific standard reference concentration. Zones sizes must fall within 3 standard deviations of the historical average. Any results greater than 2 standard deviations should be treated with caution.
- Antibiotic sensitivity patterns should be as described in the table below. Other Sensi-Disks concentrations may be used.
- Plates that do not perform as expected must be discarded and new plates produced to meet the requirements.

Table 34.7.1.3 Plate QC: Antibiotic Sensi-Disk Patterns

		Antibiotic Sensi-Disc				
Plate No. & Organism	**Agar**	**Te30**	**P10**	**S10**	**E15**	**N5**
1 - *B. cereus* ATCC 11778	# 8	S	R	S	S	S
2 - *M. luteus* ATCC 9341a	# 2 without penicillinase	S	S	R	S	R
3 - *M. luteus* ATCC 9341a	# 2 with penicillinase	S	R	R	S	R
4 - *B. subtilis* ATCC 6633	# 5	S	R	S	S	S
5 - *M. luteus* ATCC 9341a	#11	S	R	R	S	S
6 - *M. luteus* ATCC 15957	# 11	S	R	S	R	S
7 - *S. epidermidis* 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)

- Pipette the required quantity of *B. cereus* (ATCC 11778) spores into melted, tempered Antibiotic Media No. 8 to make a final concentration of 5 x 10^3 cfu/ml of agar.
- Swirl the bottle gently to mix and **incubate for 45 minutes in a 48° ± 2° C water bath**.
- After incubation add 1 ± 0.1 ml of penicillinase per 100 ml of seeded media (100,000 units per ml of agar).
- Mix the agar suspension and prepare the plates as described in Section 34.7.1.

34.7.2.2 Preparation of Plates with *M. luteus* (MLA, Plate 2)

- Pipette the required quantity of *M. luteus* cells (ATCC 9341a) into melted, tempered Antibiotic Media No. 2 to make a final concentration of 8 x 10^5 cfu/ml of agar.
- **Note: No penicillinase is added to this agar preparation.**
- Mix the agar suspension and prepare the plates as described in Section 34.7.1.

34.7.2.3 Preparation of Plates with *M. luteus* and Penicillinase (MLA+P, Plate 3)

- Pipette the required quantity of *M. luteus* cells (ATCC 9341a) into melted, tempered Antibiotic Media No. 2 to make a final concentration of 8 x 10^5 cfu/ml of agar.
- Add 1 ± 0.1 ml of penicillinase per 100 ml of seeded media.
- Mix the agar suspension and prepare the plates as described in Section 34.7.1.

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

- Pipette the required quantity of *B. subtilis* (ATCC 6633) spores into melted, tempered Antibiotic Media No. 5 to make a final concentration of 1 x 10^4 cfu/ml of agar.
- Swirl the bottle gently and **incubate for 75 minutes in a water bath at 48 ± 2°C.**
- After incubation add 1 ± 0.1 ml of penicillinase per 100 ml of seeded media (100,000 units per ml of agar).
- Mix the agar suspension and prepare the plates as described in Section 34.7.1.

34.7.2.5 Preparation of Plates with *M. luteus* ATCC 9341a and Penicillinase (MLA-11, Plate 5)

- Pipette the required quantity of *M. luteus* cells (ATCC 9341a) into melted, tempered Antibiotic Media No. 11 to make a final concentration of 8 x 10^5 cfu/ml of agar.
- Add 1 ± 0.1 ml of penicillinase per 100 ml of seeded media.
- Mix the agar suspension and prepare the plates as described in Section 34.7.1.

34.7.2.6 Preparation of Plates with *M. luteus* ATCC 15957 and Penicillinase (ML-ER, Plate 6)

- Pipette the required quantity of *M. luteus* cells (ATCC 15957) into melted, tempered Antibiotic Media No. 11 to make a final concentration of 1 x 10^5 cfu/ml of agar.
- Add 1 ± 0.1 ml of penicillinase per 100 ml of seeded media (100,000 units/ml).
- Mix the agar suspension and prepare plates as described in Section 34.7.1.

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

- Pipette the required quantity of *S. epidermidis* (ATCC 1228) cells into melted, tempered Antibiotic Agar No. 11 to make a final concentration of 1 x 10^5 cfu/ml of agar.
- Add 1 ± 0.1 ml of penicillinase per 100 ml of seeded media.
- Mix the agar suspension and prepare plates as described in Section 34.7.1.

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 of comparison 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. Track the standard curve slopes and zone sizes. If the antibiotic SR zone size changes by more than three standard deviations of historic values the curve is considered unacceptable and a new curve must be produced. Trends, such as changes in slope, high and low points, and antibiotic zone sizes, should also 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 at least 8 mm in diameter, it may be necessary to change the antibiotic standard curve concentrations in your lab so that a zone of at least 8 mm in diameter is produced at the lowest concentration.

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.

Follow the manufacturer's directions for storing and preparing the antibiotic reference standards. Use chemically clean glassware. Stock solutions, 1000 parts per million (ppm[1]), should be prepared in an atmosphere of 50% relative humidity or less. Carefully weigh a small amount of the antibiotic into a chemically clean container. Dilute the weighed powder using the appropriate Class A volumetric glassware and/or volumetric dispensing device(s) and diluent to obtain solutions of the required concentrations.

34.8.2.1 Tetracyclines

Tetracyclines are 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.

Preparation of Tetracycline and Oxytetracycline Solutions

a. Calculate the quantity of tetracycline or oxytetracycline needed to produce 1000 ppm of free base in the desired volume. Use the free base content statement provided by the manufacturer or calculate the free base content by using the molecular formula information and purity information provided by the supplier.

b. Accurately weigh the required amount of antibiotic. Add the required volume of 0.01 N hydrochloric acid to produce a 1000 ppm (ug/ml) stock solution. If the powder is transferred from the weighing container into another container for dilution, wash it into the container with 0.01 N hydrochloric acid to ensure that the entire quantity is transferred. Oxytetracycline may be slow to go into solution. It may be placed on a magnetic stirrer until dissolved or held overnight at 2-8°C.

c. Store the stock solution at 2-8°C and use it within seven (7) days.

d. Preparation of Tetracycline and Oxytetracycline Dilutions. Dilute an aliquot of the tetracycline or oxytetracycline stock solution with enough 0.1 M, pH 4.5 phosphate buffer to obtain solutions with concentrations of 1.28, 0.64, 0.32, 0.16, and 0.08 ppm. The 0.08 ppm concentration is used to indicate test

[1]Parts per million (ppm) is used in this chapter to express a quantity of 1 µg per ml.

sensitivity. The 0.32 ppm dilution is used as the SR concentration for the standard curve and for the quantitative bioassay.

The table below outlines a suggested dilution method for preparing the required concentrations of tetracycline and oxytetracycline.

From ppm Solution	Take (ml)	Dilute to (ml)	Resulting conc.*
1000.0	5.0	50	100.0
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)

Preparation of Chlortetracycline Solutions

a. Calculate the quantity of chlortetracycline needed to produce 1000 ppm of free base in the desired volume. Use the free base content statement provided by the manufacturer or calculate the free base content by using the percent purity and molecular formula information.

b. Accurately weigh the required amount of antibiotic. If the powder is transferred from the weighing container into another container for dilution, wash it into the container with 0.01 N hydrochloric acid to ensure that the entire quantity is transferred. Add the required volume of 0.01 N hydrochloric acid to produce a 1000 ppm (ug/ml) stock solution.

c. Store the stock solution at 2-8°C and use it within seven (7) days.

d. Preparation of Chlortetracycline Dilutions. Dilute an aliquot of stock solution with enough 0.1 M, pH 4.5 buffer to obtain solution concentrations of 0.16, 0.08, 0.04, 0.02, and 0.01 ppm. The 0.01 ppm concentration is used to indicate test sensitivity. The 0.04 ppm dilution is the SR concentration for the standard curve and for the quantitative bioassay.

The table below outlines a suggested dilution method for preparing the required concentrations of chlortetracycline:

From Solution (ppm)	Take (ml)	Dilute to (ml)	Resulting conc.*
1000.0	5.0	50	100.0
100.0	4.0	50	8.0
8.0	1.0	50	0.16
8.0	1.0	100	0.08
8.0	1.0	200	0.04
0.04	25.0	50	0.02
0.02	12.5	25	0.01

* In ppm (μg/ml)

34.8.2.2 Preparation of Penicillin Solutions

a. Calculate the amount of Penicillin G potassium salt needed to produce 1000 ppm of penicillin G free base in the desired volume of buffer. Use the free base content statement provided by the manufacturer for these calculations (Ex. 1). If the free base content is not available but the units content is available, calculate the quantity by converting units to micrograms (Ex. 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
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
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. If the powder is transferred from the weighing container into another container for dilution, wash it into the container with the suspension buffer to ensure that the entire quantity is transferred. Add sufficient phosphate buffer (0.1 M, pH 6.0) to make a stock solution with a final concentration of 1000 ppm (µg/ml).

c. Store the stock solution at 2-8°C and use it within seven days.

d. Preparation of Penicillin Dilutions. Dilute an aliquot of the stock solution in 0.1 M, pH 6.0 buffer using volumetric flasks to prepare working solutions with concentrations of 0.16, 0.08, 0.04, 0.02, and 0.01 ppm. The 0.01 ppm

concentration is used to indicate test sensitivity. The 0.04 ppm dilution is used as the SR concentration for the standard curve and for the quantitative bioassay.

The table below outlines a suggested dilution method for preparing the required concentrations of penicillin.

From Solution (ppm)	Take (ml)	Dilute to (ml)	Resulting Conc.*
1000	5.0	50	100
100	2.0	100	2.0
2.0	4.0	50	0.16
2.0	2.0	50	0.08
2.0	1.0	50	0.04
2.0	1.0	100	0.02
2.0	0.25	50	0.01

* In ppm (µg/ml)

34.8.2.3 Preparation of Streptomycin Solutions

a. Follow the manufacturer's instructions for use, drying a portion if necessary or calculating out the water content. Calculate the quantity of powder needed to prepare 1000 ppm of free base in the desired volume. Use the free base statement provided by the manufacturer. If the free base content is not available, calculate the quantity of powder needed for an equivalent content. Use the label potency statement (in units per mg) for the formula. Since 1 mg of pure streptomycin contains 785 units and the U.S.P. specifies not less than 650 µg and not more than 850 µg of streptomycin per mg, calculate potency of the standard by this formula:

([Potency units/mg] / [785 units/mg]) * (850 µg/mg) = Potency (µg/mg)

b. Accurately weigh the required amount of antibiotic. If the powder is transferred from the weighing container into another container for dilution, it may be

washed into the container with distilled water to ensure that the entire volume is transferred. Dilute the weighed powder with distilled water to the required volume to achieve a 1000 ppm stock solution.

c. Store the stock solution at 2-8°C and use it within seven (7) days.

d. Preparation of Streptomycin Dilutions. Dilute an aliquot of stock solution in 0.1 M, pH 8.0 phosphate buffer using volumetric flasks to prepare working concentrations of 1.6, 0.8, 0.4, 0.2, and 0.1 ppm. The 0.1 ppm concentration is used to indicate test sensitivity. The 0.4 ppm concentration is used as the SR concentration for the standard curve and in the quantitative bioassay.

The table below outlines a suggested dilution method for preparing the required concentrations of streptomycin.

From Solution*	Take (ml)	Dilute to (ml)	Resulting Conc.*
1000	5.0	50	100.0
100	2.0	50	4.0
4.0	20.0	50	1.6
4.0	10.0	50	0.8
4.0	5.0	50	0.4
4.0	2.5	50	0.2
0.2	25.0	50	0.1

* In ppm (µg/ml)

34.8.2.4 Preparation of Erythromycin Solutions

a. Use either of the 2 methods below to prepare the 1000 ppm stock solution.

- Determine the quantity of erythromycin required to prepare a 1000 ppm solution. Use the free base content of the product to calculate the amount of powder needed to make the 1000 ppm solution. Weigh this amount into a suitable container (such as a clean disposable aluminum weigh boat). Add

about 2.0 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.

or

- Weigh antibiotic powder into a container. Calculate the amount of diluent needed to prepare 1000 ppm solution of the free base. Add enough (about 2 to 3 ml) 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.

b. Store the stock solution at 2-8°C and use it within 14 days of preparation.

c. Preparation of Erythromycin Dilutions. Dilute an aliquot of the stock solution in 0.2 M, pH 8.0 phosphate buffer using volumetric flasks to prepare working concentrations of 0.8, 0.4, 0.2, 0.1, and 0.05 ppm. The 0.05 ppm dilution is the concentration used to indicate test sensitivity. The 0.2 ppm dilution is used as SR concentration on the standard curve and as the working standard in the quantitative bioassay.

The table below outlines a suggested dilution method for preparing the required concentrations of erythromycin.

From Solution*	Take (ml)	Dilute to (ml)	Resulting Conc.*
1000	5.0	50	100.0
100	2.0	50	4.0
4.0	10.0	50	0.8
4.0	5.0	50	0.4
4.0	2.5	50	0.2
0.2	25.0	50	0.1
0.1	25.0	50	0.05

* In ppm (μg/ml)

34.8.2.5 Preparation of Neomycin Solutions

a. Follow the manufacturer's instructions for use, drying a portion if necessary or calculating out the water content. Use 0.1 M, pH 8.0 phosphate buffer for preparing all solutions.

b. Determine the quantity of neomycin required to prepare a 1000 ppm solution. Use the free base content statement on the container if available. If the free base content is not available, calculate the free base from the molecular formula and percent purity. Weigh the antibiotic powder into a suitable container. If the powder is transferred from the weighing container into another container for dilution, wash it into the container with the suspension buffer to ensure that the entire quantity is transferred. Add sufficient 0.1 M, pH 8.0 phosphate buffer, to produce a concentration of 1000 ppm (µg/ml).

c. Store the stock solution at 2-8°C and use it within 30 days.

d. Preparation of Neomycin Dilutions. Dilute an aliquot of the stock solution in 0.1 M, pH 8.0 phosphate buffer using volumetric flasks to give working concentrations of 4.0, 2.0, 1.0, 0.5, and 0.25 ppm. The 0.25 ppm concentration is used to indicate test sensitivity. The 1.0 ppm dilution is the SR concentration on the standard curve and the working standard in the quantitative bioassay.

The table below outlines a suggested dilution method for preparing the required concentrations of neomycin.

From Solution*	Take (ml)	Dilute to (ml)	Resulting Conc.*
1000	10	100	100.00
100	10	100	10.0
10	4	10	4.0
10	2	10	2.0
10	1	10	1.0
10	0.5	10	0.5
10	0.25	10	0.25

* In ppm (µg/ml)

34.8.3 Standard Curves

34.8.3.1 Procedure

a. Use prepared plates that have met all QC requirements. 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 $200 \pm 4\mu l$ 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.

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 Calculations

Calculate the corrected standard curve and linear regression. This may be done manually or using an appropriate validated computer program. The manual method is described in the Appendix 34A.5, Manual Calculation of a Standard Curve.

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.

Calculate cumulative average zones for all SR concentrations on the entire set of 12 plates.

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.

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.

34.9 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. The laboratory should take measures to ensure good housekeeping. 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® filter bags, Whirl-Pak® 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 frozen, homogenized tissue portion may be used for up to 14 days after preparation. 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 ± 0.2 g of tissue (avoid fat) into each bag. If a small amount of sample (i.e. < 100g) is available, use 5 ± 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.

It is suggested that the sample bags be set up in a manner to prevent confusion and facilitate sample preparation. An example of how to separate the bags into racks by pH of the buffer is shown below.

Rack No. 1 (Buffer pH 4.5)	Rack No. 2 (Buffer pH 6.0)	Rack No. 3 (Buffer pH 8.0, 0.1M)	Rack No. 4 (Buffer pH 8.0, 0.2M)
Muscle	Muscle	Muscle	Muscle
Kidney	Kidney	Kidney	Kidney
Liver	Liver	Liver	Liver

d. For 10 gram samples, dispense 40 ± 1.0 ml of the appropriate buffer into each bag. For 5 gram samples, use 20 ± 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. Reserve tissues should be held for the time prescribed by the laboratory.

34.9.2 Preparation of Spiked Tissues

In order to validate a new assay or changes to an assay, to detect the presence of a new antibiotic in tissue, or 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.

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.

c. Follow the extraction procedures (Section 34.9.1) 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). 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 screening test may be done to determine the type of antimicrobial residue present, followed by the appropriate quantitative analysis. Alternatively, the full seven-plate bioassay may be done on the sample(s), accomplishing both identification and quantitation at the same time. If the antimicrobial residue present is already known, the quantitative step may be performed to determine the concentration of the residue. Each of these 3 approaches is described below. The individual

assays are described in Section 34.10. Instructions on reading the plates are given in Section 34.11. Interpretation of results is found in Section 34.12.

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.

34.9.3.1 General Instructions

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	MLA	(beta-lactam detection – pH 6.0 buffer extract)
Plate 3	MLA+P	(penicillin confirmation – pH 6.0 buffer extract)
Plate 4	BS	(streptomycin detection – pH 8.0, 0.1M buffer extract)
Plate 5	MLA-11	(macrolide detection – pH 8.0, 0.2M buffer extract)
Plate 6	MLER	(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:

- 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. The well below the orientation hole designates the starting point.
- Fill the wells with 200 ± 4 µl of the appropriate test sample extract or SR.
- Place the lids on the plates and incubate plates 1 through 6 at 29 ± 1°C for 16 to 18 h. Incubate plate 7 at 37 ± 1°C for 16-18 h.
- 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.

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), 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. Fill one well on each of the seven plates with 200 ± 4 µl 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 gentamycin. Pipette 200 ± 4 µl of the SR concentration into the test well. Sensi-Discs may be used on a separate plate in place of the reference dilutions.

d. Incubate as described in Section 34.9.3.1.

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), 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, fill three alternate wells with 200 ± 4 µl of the appropriate buffered sample extract. Fill the other 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.

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 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, fill three alternate wells with 200 ± 4 µl 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.

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 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 role of the bioassay in some instances is simply to indicate the presence of a class of antibiotic.

34.10.1 Tetracyclines – 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.

34.10.1.1 Performing the Assay:

- Use Plate 1 (prepared with antibiotic medium No 8, *Bacillus cereus* ATCC 11778 and penicillinase as described in Section 34.7.2.1).
- Add 200 ± 4 µl of the sample extracted in 0.1M, pH 4.5 buffer to the test wells.
- Tetracycline is the standard reference antibiotic for this plate.
- Incubate the plates at 29 ± 1°C for 16 to 18 hours.

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

The tetracyclines are tentatively identified by zones of inhibition on BC Plate 1. When the concentration of tetracycline is low, there may be no zones of inhibition (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. Other appropriately validated methods may also be used.

34.10.1.3 Quantitation of Tetracycline in Tissue

After the residue is identified, perform a quantitative analysis on 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.

34.10.2 Penicillin and antibiotics giving the penicillin pattern – Plates 2 and 3

34.10.2.1 Performing the assay:

- Use Plate 2 (prepared with *M. luteus* ATCC 9341a, antibiotic medium No. 2 without penicillinase) and Plate 3 with penicillinase (prepared as described in Section 34.7.2.2. and 34.7.2.3).
- Add 200 ± 4 µl of the sample extracted in 0.1M, pH 6.0 buffer to the test wells.
- Penicillin G potassium salt is the reference antibiotic for these plates.
- Incubate the plates at 29 ± 1°C for 16 to 18 hours.

34.10.2.2 Tentative Identification of Penicillin and/or Penicillin-Like Residues in Tissue from the Seven Plate Bioassay System

The presence of penicillin in a sample is indicated by zones of inhibition on Plate 2 and no zone on Plate 3. The identity of residues matching this plate pattern may be confirmed by additional testing.

34.10.2.3 Quantitation of Penicillin or Penicillin-Like Residues in Tissue

Upon identification of the penicillin-like 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 of other penicillin-like antibiotics include ampicillin and amoxicillin. See Table 34.14 (Standard Curve Summary Table) for additional antibiotics.

34.10.3 Streptomycin and Streptomycin-like Antibiotics - Plate 4

34.10.3.1 Performing the assay:

- Use plate 4 prepared with *B. subtilis* ATCC 6633 and antibiotic medium No. 5 (See Section 34.7.2.4).
- Add 200 ± 4 µl of the sample extracted in 0.1 M, pH 8.0 buffer to the test wells.
- Streptomycin is the reference antibiotic for this plate.
- Incubate the plates at 29 ± 1°C for 16 to 18 hours.

34.10.3.2 Tentative Identification of Streptomycin in Tissue from the Seven Plate Bioassay System

The presence of streptomycin is tentatively identified from zones of inhibition on BS Plate 4. There will be no zones on any other plates if streptomycin is in low concentration. If it is in high concentration, there may be zones on Plates 1 and Plate 6. There should be no zones of inhibition on Plates 2, 3, 5, and 7. Identification must be confirmed by other methods. Dihydrostreptomycin, spectinomycin, and low concentrations of fluoroquinolones may produce similar patterns. The specific antibiotic must be confirmed by chemical tests.

34.10.3.3 Optional: Use of an Additional Plate with a Resistant Organism for Streptomycin Confirmation

Use plates prepared with Antibiotic Medium No. 5, M. *luteus* ATCC 9341a (which is resistant to streptomycin), and penicillinase to test the sample extracted in 0.1 M, pH 8.0 buffer. This plate is prepared in the same manner as the other *M. luteus* plates. Add sample and standard reference antibiotic as described in Section 34.10.3.1. There should be no zone of inhibition produced by the sample or the streptomycin SR.

34.10.3.4 Quantitation of Streptomycin in Tissue

Upon confirmation of streptomycin, perform quantitative analysis using 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.

NOTE: At times, both streptomycin and penicillin may be present in the same tissue. In that case, there will be zones of inhibition on Plates 2 and 4. Measure the zones and calculate the antibiotic contents of the sample for penicillin from Plate 2 and for confirmed streptomycin from Plate 4.

34.10.4 Erythromycin – Plate 5 and 6

34.10.4.1 Performing the assay:

- Use Plate 5 (prepared with *M. luteus* ATCC 9341a, and Antibiotic Agar No. 11), and Plate 6 (prepared with *M. luteus* ATCC 15957 and Antibiotic Agar No. 11) (Sections 34.7.2.5 and 34.7.2.6).
- Add 200 ± 4 µl of the sample extracted in 0.2 M, pH 8.0 buffer to the test wells.
- Erythromycin is the reference antibiotic for this assay.
- Incubate the plates at 29 ± 1°C for 16 to 18 hours.

34.10.4.2 Identification and Confirmation of Erythromycin in Tissue from the Seven Plate Bioassay System

The presence of erythromycin in tissue is indicated by zones of inhibition on MLA-11 Plate 5 and no zones of inhibition on MLER Plate 6. Use the zone size on Plate 5 and the appropriate standard curve to calculate the quantity of erythromycin residue present in the sample as described in Section 34.13.

34.10.4.3 Quantitation of Erythromycin in Tissue

Upon confirmation of erythromycin, use the sample zone size on Plate 5, the erythromycin SR zone size, and the appropriate standard curve to calculate the quantity of erythromycin in the sample.

34.10.5 Neomycin – Plate 7

34.10.5.1 Performing the assay:

- Use Plate 7 (prepared with antibiotic agar No. 11, *S. epidermidis* ATCC 12228 and penicillinase as described in Section 34.7.2.7).
- Add 200 ± 4 µl of the sample extracted in 0.1 M, pH 8.0 buffer to the test wells.

- Neomycin is the reference antibiotic for this assay.
- Incubate the plates at 37 ± 1°C for 16 to 18 hours.

34.10.5.2 Identification of Neomycin in Tissue from the Seven Plate Bioassay System

Neomycin residue present in tissue at low concentrations (<1.0 µg/g) produces zones of inhibition only on Plate 4 and 7. The largest zones of inhibition are usually observed on Plate 7. At higher concentrations, neomycin may also produce zones of inhibition on additional plates, even all 7 plates. Identification of neomycin must be confirmed using additional tests. Other macrolides and aminoglycosides, such as tylosin and gentamicin, may produce similar patterns. Chemical tests must be used to establish the identity of the antibiotic.

34.10.5.3 Quantitation of Neomycin in Tissue

Use the sample zone size on 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. This plate is also used to calculate the quantity of gentamicin residue in tissue using the gentamicin SR dilution as described in the Summary Table in 34.14.

34.11 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 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.

b. Arrange test plates according to numerical sequence of the plates.

c. Align the zone reader at ‘0’ and, starting clockwise from the first well (marked line), read the zone of inhibition using the dial on the zone reader. Alternatively, calipers 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.

34.12 Additional Information on Test Interpretation

To identify an antibiotic, the pattern of the zones of inhibition produced by a sample should be compared with the patterns produced by known antibiotics. The presence and/or absence of zones of inhibition 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 34.15 “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.

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

   Example 1:
   The average zone size of the SR concentration on the standard curve = 20.5
   The average zone size of the SR concentration on the test plate = 19.1
   The difference = 1.4

   The average zone size of the test sample = 17.3.
   Add 1.4 to 17.3 = adjusted zone size of 18.7. 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

The average zone size of the SR concentration on the test plate = 21.1
The difference = –0.6

The average zone size of the test sample = 17.3.
Add '–0.6' to 17.3 = adjusted zone size of 16.7. 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.

- 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.
- 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).
- 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.
- Use the appropriate current standard curve in your calculations. Round off the calculated concentration to the nearest hundredth microgram.

34.14 Summary of Antibiotic Reference Standards

In order to assist in the antibiotic bioassay, a summary table is presented, describing appropriate antibiotic plates, microorganisms, buffers, and antibiotic standard concentrations necessary to prepare standard curves for many antimicrobials with or without an established antibiogram pattern. A standard curve prepared by using these specifics 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. A key to the bioassay plate composition is found below the table. Note that Plates 3 and 6 are confirmatory-type plates and are not used for standard curves.

Standard Curve Summary Table

Antimicrobial	Bioassay Plate*	Antibiotic Buffer Diluent	Concentrations (µg/ml) used for the Standard Curve
Ampicillin	Plate 2, MLA	0.1 M, pH 6.0	0.01, 0.02, **0.04**, 0.08, 0.16
Bacitracin	Plate 2, MLA	0.1 M, pH 6.0	0.2, 0.4, **0.8**, 1.6, 3.2
Chloramphenicol	Plate 2, MLA	0.1 M, pH 6.0	4.0, 8.0, **16.0**, 32.0, 64.0
Chlortetracycline [iii]	Plate 1, BC	0.1 M, pH 4.5	0.01, 0.02, **0.04**, 0.08, 0.16
Cloxacillin	Plate 2, MLA	0.1 M, pH 6.0	0.32, 0.64, **1.28**, 2.56, 5.12
Erythromycin [i]	Plate 5, MLA-11	0.2 M, pH 8.0	0.05, 0.1, **0.2**, 0.4, 0.8
Flavomycin [ii]	Plate 1, BC	0.1 M, pH 4.5	0.05, 0.1, **0.2**, 0.4, 0.8
Gentamicin	Plate 7, SE	0.1 M, pH 8.0	0.15, 0.25, **0.5**, 1.0, 1.5
Hygromycin	Plate 4, BS	0.2 M, pH 8.0	5.0, 10.0, **20.0**, 40.0, 80.0
Lincomycin	Plate 5, MLA-11	0.2 M, pH 8.0	0.3, 0.6, **1.2**, 2.4, 4.8
Neomycin	Plate 7, SE	0.1 M, pH 8.0	0.25, 0.5, **1.0**, 2.0, 4.0
Novobiocin	Plate 7, SE	0.2 M, pH 8.0	0.25, 0.5, **1.0**, 2.0, 4.0
Oleandomycin	Plate 5, MLA-11	0.2 M, pH 8.0	0.25, 0.5, **1.0**, 2.0, 4.0
Oxytetracycline [iii]	Plate 1, BC	0.1 M, pH 4.5	0.08, 0.16, **0.32**, 0.64, 1.28
Penicillin	Plate 2, MLA	0.1 M, pH 6.0	0.01, 0.02, **0.04**, 0.08, 0.16
Spectinomycin	Plate 4, BS	0.2 M, pH 8.0	10, 20, **40**, 80, 160
Streptomycin [iv]	Plate 4, BS	0.1 M, pH 8.0	0.1, 0.2, **0.4**, 0.8, 1.6
Sulfamethazine	Plate 5, MLA-11	0.2 M, pH 8.0	30, 60, **120**, 240, 480
Tetracycline [iii]	Plate 1, BC	0.1 M, pH 4.5	0.08, 0.16, **0.32**, 0.64, 1.28
Tilmicosin [i]	Plate 5, MLA-11	0.2 M, pH 8.0	0.1, 0.2, **0.4**, 0.8, 1.6
Tylosin	Plate 5, MLA-11	0.2 M, pH 8.0	0.2, 0.4, **0.8**, 1.6, 3.2
Virginiamycin	Plate 5, MLA-11	0.2 M, pH 8.0	1.28, 2.56, **5.12**, 10.24, 20.48

i. Dissolve the antibiotic powder in a small volume of methanol or 30% methanol before diluting it in the phosphate buffer solution.
ii. Dissolve the antibiotic powder in a small volume of methanol or 50% methanol before diluting it in the phosphate buffer solution.
iii. Dissolve the tetracycline powder in 0.01 N hydrochloric acid, to make the 1000 ppm solution, before preparing the dilutions.
iv. Dissolve streptomycin powder in distilled water to make the 1000 ppm solution before preparing dilutions.

*Bioassay Plate Identity	Plate Composition
Plate 1, BC	*B. cereus* ATCC 11778 in Antibiotic Medium 8
Plate 2, MLA	*M. luteus* ATCC 9341a in Antibiotic Medium 2
Plate 4, BS	*B. subtilis* ATCC 6633 in Antibiotic Medium 5
Plate 5, MLA-11	*M. luteus* ATCC 9341a in Antibiotic Medium 11
Plate 7, SE	*S. epidermidis* ATCC 12228 in Antibiotic Medium 11

34.15 Additional Testing Procedures

34.15.1 ELISA Tests

Enzyme-Linked Immunosorbent Assay (ELISA) tests may be used as screen tests or to identify the antibiotic residues present in a sample. ELISA tests for sulfonamides are used for screening suspect tissues. ELISA tests for gentamicin, neomycin, and tylosin are currently used to identify residues that give bioassay patterns indicative of these antibiotics. Commercially available tests may be used. If the test is not designed for use with tissue extracts, it must be validated for the tissues with which it will be used. Non-commercial tests may be used with appropriate validation or equivalency studies as prescribed by the laboratory's operating procedures. All ELISA tests are validated for the intended application before they are used. In addition, each lot must pass lot acceptance criteria. Lot acceptance criteria may be found in the laboratory SOPs. Positive and negative tissue controls must always be used when running ELISA tests.

34.15.2 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. Tissues that give a pattern indicative of the presence of aminoglycosides can be tested to identify the antibiotic present using the LC/MS/MS method CLG-AMG1. Amikacin, apramycin, dihydrostreptomycin, gentamycin, hygromycin, kanamycin, neomycin, spectinomycin, streptomycin, and tobramycin are among the antibiotics that can be identified by this method. Macrolides and

lincosamides can be identified using the LC/MS/MS method CLG-MAL, including clindamycin, erythromycin, lincomycin, pirlimycin, tilmicosin, and tylosin. Tilmicosin can also be identified using method CLG-TIL2, and quantified using method CLG-TIL1. Many sulfonamide residues can be identified using the method CLG-SUL.

Additional chemical methods of identifying and quantitating antibiotic residues may also be available.

34.16 References

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34.A Appendix

34.A.1 General Information

The material in this appendix is included primarily for informational purposes. FSIS laboratories do not routinely use these procedures. Some of the materials and techniques may be outdated. The information is included for reference. However, these procedures (or similar procedures) may be used when needed.

34.A.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. 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.A.3 Preparation, Storage, and Use of Vegetative Cell Cultures for Assay

34.A.3.1 Equipment, Materials, Reagents, and Media (for 34.A.3 and 34.A.4)

Equipment and Materials

a. Bausch & Lomb Spectronic 20 or comparable spectrophotometer
b. Refrigerator, 2 to 8 °C
c. Freezer capable of maintaining –15°C
d. Incubators capable of maintaining temperatures of 29 ± 1°C, 35 ± 1°C, and 37 ± 1°C
e. Water bath, 40 to 48°C range with ± 2°C of set temperature
f. Colony counter
g. Analytical balance
h. General laboratory balance 0.1 to 200 g capacity
i. Microscope, at least 400X magnification
j. Centrifuge, super speed
k. Centrifuge, refrigerated, 1500 to 5000X gravity capacity
l. Magnetic Stirrer – Heated
m. Vortex mixer

n. Roux Bottles (optional)
o. Sterile glass beads
p. Homogenizer or blender
q. Virtis jars or homogenizer jars
r. Centrifuge tubes, 50 ml
s. Tubes with caps, 12 x 75 mm plastic storage type
t. Assorted tubes, graduated cylinders volumetric flasks, volumetric pipettes
u. Petri dishes 100 x 15 mm
v. Erlenmeyer flasks, 250 ml
w. Semi-logarithmic graph paper
x. Autoclave

Reagents and Media

a. Hydrogen Peroxide 3%
b. Glycerol
c. Methylcellulose (See Section 34A.3.5.3b)
d. Dehydrated Alcohol, Ethyl Alcohol, 200 Proof, or 50% ethyl alcohol in purified water
e. 3 M, pH 7.1 phosphate buffer (recipe in Section 34.A. 4.2 e.) or density gradient forming agents, such as Percoll
f. Polyethylene glycol
g. A-K Agar # 2 (Sporulating Agar) with extra 0.5% purified agar
h. Blood agar plates (Sheep blood in Tryptose Soy Agar)
i. Plate count agar (PCA)
j. Antibiotic Media No's 1, 8, and 11
k. Standard reference antibiotics: penicillin, erythromycin, neomycin, and streptomycin
l. Brain Heart Infusion Broth regular strength and 2X (double strength)
m. Tryptose soy agar (TSA)
n. Gram stain reagents
o. Sterile physiological saline (0.85% NaCl)
p. Biochemical identification systems such as API Staph Trac, VITEK, MicroId, or biochemical reagents as needed to identify the microorganisms per SOP LW-0015:
q. Phenol Red Dextrose Broth (optional)
r. Phenol Red Mannitol Broth (optional)
s. Phenol Red Lactose Broth (optional)
t. Trypticase Soy Agar slants or plates (TSA)
u. Butterfield's phosphate buffer
v. Glycerol- saline (See Section 34.A.3.5.3)
w. Sodium chloride and magnesium sulfate for preparation of reagents listed above

x. O/F glucose broth
y. Methyl Red/Voges-Proskauer (MR/VP) test broth
z. Mannitol broth
aa. Malachite green stain
bb. Safranin stain
cc. Mannitol Egg Yolk Polymyxin (MYP) Agar (Difco agar base with 50% egg yolk enrichment, and antimicrobic vial P) (Optional)
dd. Motility Medium

34.A.3.2 Test Organisms

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. *Micrococcus luteus,* ATCC 9341a (MLA)
b. *Micrococcus luteus,* ATCC 15957 (MLER)
c. *Staphylococcus epidermidis,* ATCC 12228 (SE)
d. *Bacillus cereus* var. *mycoides* spores, ATCC 11778 (BC)
e. *Bacillus subtilis* spores, ATCC 6633 (BS)

34.A.3.3 Determination of Purity and Properties of Vegetative Test Organisms

34A.3.3.1 Purity and Identity

All cultures must be confirmed to be pure and their identity biochemically confirmed before they are acceptable for use. Manufacturer's certificates are acceptable for commercial preparations. Laboratory tests for purity and biochemical confirmation may be done as described below. Appropriate commercial biochemical test kits may be used in place of the traditional biochemical tests described. Before preparing cell suspensions from fresh or lyophilized cultures for assay, use a portion of each test organism to determine its purity and other important characteristics as follows:

a. Streak two plates of tryptose soy agar each with M. *luteus* ATCC 9341a (MLA), *M. luteus* ATCC 15957 (ML-ER), and *S. epidermidis* ATCC 12228 (SE), streaking for isolation. Incubate the plates overnight at 35°C to 37°C.

b. Examine the plates for purity. Check the color of colonies. Colonies of both strains of *Micrococcus* are yellow, and colonies of *Staphylococcus* are white. Prepare a Gram stain of each test organism. All cultures are Gram positive. *Micrococcus* spp. usually appear as cocci in packets of eight. *S. epidermidis* appear as cocci in irregular clusters.

c. Check biochemical reactions of the cultures. Use one TSA plate of each organism to test for presence of catalase. Place a drop of hydrogen peroxide on the plate and observe for bubble formation. Generation of bubbles indicates a positive catalase test. All organisms are catalase positive. Select isolated colonies from the second TSA plate and inoculate dextrose, lactose, and mannitol broths. Incubate at 35 ± 1°C overnight. The biochemical patterns should be as follows:

Organism	Dextrose	Lactose	Mannitol	Catalase
SE	+	-	-	+
MLA	-	-	-	+
MLER	-	-	-	+

34.A.3.3.2 Determination of Antibiotic Sensitivity

All organisms must be tested to confirm antibiotic sensitivity. Prepare bioassay plates listed in the table below as described in Section 34.7 **with the exception that penicillinase should not be added to the agar.** Place a 'spider' or 5 cylinders on each plate. Add 200 microliters of the antibiotic concentrations specified in the table below to individual wells. Each prepared plate will be tested with all four of the antibiotics listed in the table. 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.

Test Organism	Medium No.	Antibiotic concentration in mg per ml				
		Erythromycin	Neomycin	Penicillin	Streptomycin	Tetracycline
M. luteus 9341a	2	0.05	5.0	0.0125	> 200	0.5
M. luteus 9341a	11	0.025	1.5	0.0125	250	0.4
M. luteus 15957	11	> 200	1.5	0.0125	1.0	0.2
S. epidermidis 12228	11	0.075	0.25	0.8	1.5	250
B. cereus 11778	8	0.25	2.00	20	1.00	0.08
B. subtilis 6633	5	0.04	0.25	0.0125	0.2	0.08

34.A.3.4 Propagation of Test Organisms (Non-spore Formers)

This procedure is used when cultures are transferred on a weekly basis instead of using preserved cultures.

a. Open a vial of each lyophilized culture: *Micrococcus luteus* ATCC 9341a (MLA), *M. luteus* ATCC 15957 (MLER), and *S. epidermidis* ATCC 12228 (SE). Suspend the organisms in separate tubes of Brain Heart Infusion broth.

b. Incubate *M. luteus* ATCC 9341a (MLA) and *M. luteus* ATCC 15957 (MLER) at 29 ±1°C for 18 h and *S. epidermidis* ATCC 12228 (SE-TR/SE-SR) at 37 ± 1°C for 24 h.

c. After incubation, inoculate duplicate slants of Antibiotic medium # 1 with *M. luteus* ATCC 9341a, *M. luteus* ATCC 15957, and *S. epidermidis* ATCC 12228. Incubate all *M. luteus* slants at 29 ± 1°C for 18-24 h and *S. epidermidis* slants at 37 ± 1 °C for 18-24 h.

d. Store the slants at 4-8°C and transfer cultures to fresh slants every other week. Use the same procedures described in step ‘c.’ above for culture transfers.

34.A.3.5 Preparation and Preservation of Stock Cultures

Cultures may be preserved for long term storage instead of transferring them on a weekly basis, or using commercial preparations as described in 34.6.1

34.A.3.5.1 Preparation of Batch Cultures for Preservation

If results agree with criteria in Section 34.A. 3.3 at the next culture transfer:

a. Inoculate each culture onto two agar slants of the appropriate media described in 'b' below. Incubate 18-24 h at 35 to 37°C.

b. Wash the growth from each slant with 2 ml of sterile normal saline and transfer the suspension to the surface of each of two Roux bottles containing 300 ml of the proper media.

   i. Use Antibiotic Medium No.1 for *M. luteus* ATCC 9341a and *S. epidermidis* ATCC 12228.

   ii. Use Antibiotic Medium No. 11 for *M. luteus* ATCC 15957.

c. Spread the suspension evenly over the entire agar surface with the aid of sterile glass beads.

d. Incubate for 18-24 h at 35 to 37°C.

e. Wash growth from the agar surface of each Roux bottle with 40 ml of sterile saline. (There are 2 Roux bottles for each culture. Keep each Roux bottle suspension separate at this point.) Place each suspension in a 50 ml centrifuge tube, and centrifuge at 5000 x G for 30 minutes at 10 ± 2°C.

f. Pour off the supernatant, resuspend each pellet in 40 ml sterile saline, and centrifuge as above. Repeat one more time.

g. After the final centrifugation, pour off the supernatant and use 10 ml of sterile saline to resuspend the pellet from each tube of the three bacterial cultures. Pool together only the identical bacterium from the duplicate Roux bottles for a total

of 20 ml of stock culture each. Do not mix the bacteria. Pool bacteria of the same strain only.

34.A.3.5.2 Determining Number of Colony Forming Units (CFU)

Follow the procedures described in Section 34.6.3 to determine the number of bacteria per milliliter in each fresh culture suspension.

34.A.3.5.3 Preservation of Vegetative Cultures

A procedure for preserving vegetative cultures *in situ* for readily preparing assay plates has been developed. The procedure described here is simple and reliable for storing standardized, non-spore-forming organisms for up to one year. The method is as follows:

a. Preparation of Glycerol-Saline Solution

- Add 8.5 g NaCl to 850 ml of distilled water.
- Mix on a magnetic stirrer until the solution is clear.
- Add 150 ml of glycerol and mix until clear.
- Autoclave for 15 minutes at 15 lbs. pressure (121°C). The glycerol-saline mixture is now ready for use.

b. Preparation of Methylcellulose Solution

- Weigh out 12 g $MgSO_4$ and dissolve in 1 liter of distilled water.
- Place the solution on a heated magnetic stirrer.
- Add, with stirring, 10 g of methylcellulose and slowly bring to a boil.
- Boil 5-10 minutes until small amorphous aggregates of methylcellulose are formed.
- Immediately autoclave for 15 minutes at 15 lbs. pressure and 121°C. On cooling, the methylcellulose disperses and stays in solution and is ready for use.

c. Aseptically pipette 15 ml of each stock culture containing at least 5×10^8 cfu/ml into the sterile 250 ml Erlenmeyer flask and add 135 ml of either the glycerol-saline or the methylcellulose solution.

d. Prepare racks containing 140, properly identified and labeled, 12 x 75 mm plastic storage tubes with caps.

e. Mix the flask contents on a magnetic stirrer and then aseptically pipette 1 ml from the flask into each tube.

f. Close the tube caps securely.

g. Store properly labeled cultures according to conditions described in 34.A.3.5.4.

34.A.3.5.4 Shelf Life of Preserved Cultures

The prepared tubes may be handled according to the following chart:

Organism	Storage Time	Preserving Fluid	Storage Temperature
MLA, MLER	1-5 months	Glycerol-Saline	4°C
MLA, MLER & SE	1-5 months	Glycerol-Saline	-14°C
MLA, MLER & SE	1 year	Methyl-Cellulose	-14°C

34.A.3.5.5 Reconstituting Preserved Vegetative Organisms for Use

a. Thaw preserved methylcellulose or glycerol-saline preserved cultures slowly at room temperature.

b. Vortex until the suspension appears smooth.

c. Warm the suspension in a 40°C water bath, vortex again or aspirate eight times up and down using a 1 ml pipette. Inoculate the appropriate melted agar medium with the required volume of the suspension based on stock cfu and desired cfu per plate.

d. If the culture is used for other tests, then pipette the remaining portion into a 3 ml tube of 2x Brain Heart Infusion Broth. Incubate the tube at 29°C for 18-24 h.

34A.3.6 Use of Fresh Vegetative Cultures in Assay

If preserved cultures are not available, fresh organisms from slants may be prepared and used for bioassay plates. The method described here is an AOAC recommended procedure for antibiotic assays. However, the procedure is subjective and thus does not allow finer adjustment in organism concentration to achieve desired assay sensitivity, which can be better achieved by making dilutions of cell suspensions of known cfu/ml.

a. Maintain cultures by transferring onto Antibiotic Agar No. 1 slants as described in Section 34.A.3.4.

b. Inoculate the surface of duplicate slants of Antibiotic medium # 2 with *M. luteus* ATCC 9341a, and Antibiotic medium # 11 slants with *S. epidermidis* ATCC 12228 and *M. luteus* ATCC 15957. Incubate all *M. luteus* slants at 29°C for 18-24 h and S. *epidermidis* slants at 37°C for 18-24 h.

c. Turn on the spectrophotometer to warm it up. Set it to 580 nm. Transfer 2 ml of sterile 0.85% NaCl solution (saline) onto each fresh agar slant culture. Emulsify the cell suspension thoroughly, taking care not to scratch the agar.

d. Pipette 1 ml of cell the suspension into 15 ml of saline. Vortex the new mixture. Blank the spectrophotometer with saline to 100% transmittance. Adjust the percent transmittance of the *M. luteus* suspensions to 14% and the transmittance of the *S. epidermidis* suspension to 40% by adding saline or additional culture suspension as needed.

e. Add 0.5 ml of each cell suspension to the appropriate antibiotic medium in 100 x 15 mm Petri dishes to make assay plates. Do not use these cell suspensions more than 1 week.

f. If the bioassay plates appear contaminated, discard the cell suspension. Check the stock cultures, or purchase a new culture from ATCC.

34.A.4 Preparation of Suspensions of Spore Forming Test Organisms

B. cereus ATCC 11778 and *B. subtilis* ATCC 6633 may be maintained on agar by weekly transfers. Alternatively spore suspensions may be prepared for long term storage preserved in 50% ethanol. To maintain cultures, grow *B. cereus* on slants of Antibiotic Medium No. 8. Incubate at 29 to 30°C overnight. Grow *B. subtilis* on slants of Antibiotic Medium No. 1. Incubate at 35 to 37 °C overnight. Store the cultures between transfers at 2-8°C.

34.A.4.1 Determination of Purity and Properties of Spores.

Before preparing spore suspensions for long-term storage, the purity and properties of the cultures must be confirmed. If the cultures are carried by regular subculture on agar slants, the purity and properties must also be confirmed at regular intervals as described in the laboratory quality system. To perform biochemical confirmation of either organism, use appropriate, recognized commercial test kits or follow the biochemical procedures described below.

a. If a lyophilized culture is used, reconstitute it in Brain Heart Infusion (BHI) broth. Streak the suspension onto two plates of Tryptose Soy agar with 5% defibrinated sheep blood for isolation using a loopful of culture from the BHI broth or from the regular agar slant culture. Inoculate a Brain Heart Infusion agar slant at the same time. The optional MYP agar plate and motility medium may also be inoculated at the same time. Incubate all cultures overnight at the appropriate temperatures.

   Examine the Blood Agar plates for purity. Note the size and type of hemolytic zone. *B. cereus* should produce a zone of beta hemolysis around the colonies. *B. subtilis* should not produce a zone of hemolysis.

b. Prepare a Gram stain and Malachite Green stain from three well-isolated colonies. Counter-stain the Malachite green slide with Safranin. The cells should be Gram positive. On the slide stained with Malachite green and Safranin spores will appear green and the vegetative cells will be red or pink. Endospores should be oval and centrally located. The spore should not distend the vegetative cell.

c. Use the BHI slant to test for catalase activity. Both organisms should be catalase positive. (A positive test is production of bubbles when a drop of hydrogen peroxide is added to the cell growth.)

d. Inoculate O/F glucose, Methyl Red/Voges-Proskauer (VP), and mannitol broths with isolated colonies from the blood agar plate. Incubate overnight and record results. The mannitol broth is not required if the MYP agar plate is used. They should agree with the following chart:

Organism	β hemolysis	O/F glucose	Mannitol	MR/VP
B. cereus	Positive	Oxidative & Fementative	Negative	Negative/Positive
B. subtilis	Negative	Oxidative	Positive	Positive/Positive

MYP and Motility Agar Results

Organism	MYP lecithinase	MYP mannitol	Motility
B. cereus	zone of precipitation	Negative (pink)	Positive
B. subtilis	No zone	Positive (yellow)	Positive

Any organism that is not pure or does not meet the biochemical identification listed should be replaced. **NOTE**: Any organism that is non-motile, lecithinase positive, mannitol negative, and non-hemolytic is possibly *Bacillus anthracis*. This organism is potentially lethal to humans. Stop work with this culture and thoroughly disinfect all work surfaces with 0.5% sodium hypochlorite for at least 15 minutes.

34.A.4.2 Preparation of Spore Suspensions of *Bacillus cereus* ATCC 11778

a. Reconstitute a lyophilized culture of *B. cereus* ATCC 11778 in Brain Heart Infusion broth and incubate overnight at 35 to 37°C.

b. Transfer several well isolated colonies from the blood agar plate onto A-K Sporulating Agar # 2 slants with extra 0.5% Purified Agar (Difco or equivalent) and incubate at 37°C.

c. Add 4 to 6 sterile glass beads and 2 to 3 ml of sterile distilled water to the agar slants. Shake the slants gently for 2 minutes to dislodge the bacterial growth. Aseptically pipette the bacterial suspension into a Roux bottle containing 300 ml of A-K Sporulating Agar # 2 with extra 0.5% Purified Agar (Difco or equivalent) and spread over the surface. Multiple cultures may be prepared and pooled for transfer to Roux bottles.

Incubate the Roux bottles horizontally at 35-37°C for 18 to 24 h, then incubate at room temperature for the remainder of 1 week (6 days).

d. Harvest the growth from the Roux bottles by adding 20-30 sterile glass beads and approximately 25 ml of sterile distilled water per bottle. Gently agitate the bottle to dislodge the bacterial growth. Care must be taken not to break the agar during harvesting. Aseptically transfer this suspension to sterile centrifuge tubes (40 ml volume) and heat for 10 minutes in boiling water at 100°C. Wash the spore suspension three times with sterile distilled water by centrifuging and decanting in the following manner:

   i. Centrifuge at 5°C for 20 minutes at 27,000 x G.
   ii. Pour off supernatant.
   iii. Resuspend the pellet in 20 ml sterile distilled water.
   iv. Repeat Steps i, ii, and iii two more times.

e. The spores may be further purified using density gradient centrifugation. The method described here or other appropriate method may be used.

   Prepare 3 M, pH 7.1 phosphate buffer.
   K_2HPO_4 306.9 g
   KH_2PO_4 168.6 g
   0.1 N HCl or 0.1 N NaCl

   Dissolve the potassium salts in distilled water. Adjust the pH with hydrochloric acid or sodium hydroxide if necessary to a final pH of 7.1. Bring the volume to 1.0 liter. Filter sterilize the solution.

f. Using 3 M, pH 7.1 phosphate buffer, wash and coat a sterile Virtis jar in the following manner:

   Combine 34.1 ml of sterile 3 M, pH 7.1 phosphate buffer and 11.8 g of sterile polyethylene glycol in a sterile 100 ml glass stoppered graduated cylinder or volumetric flask. Shake vigorously. Bring to volume with sterile distilled water. Pour the mixture into a sterile Virtis jar and place the jar on the homogenizer. Blend for 5 minutes at 5,000 RPM. Discard the mixture. Repeat.

g. Combine 34.1 ml of sterile 3 M, pH 7.1 phosphate buffer and 11.8 g of sterile polyethylene glycol in a sterile 100 ml glass stoppered graduated cylinder or volumetric flask. Add 25 ml of the washed spore mixture and bring to volume with sterile distilled water. Shake vigorously. Pour the mixture into the coated Virtis jar and homogenize for 5 minutes at 5,000 RPM.

h. Dispense the resulting mixture equally into four sterile centrifuge tubes and centrifuge in a swinging bucket rotor at 1,500 x G for 2 minutes at room temperature.

i. A two-phase system with an interface will be formed in the centrifuge tube. The spores are in the upper phase, sub-cellular debris collects at the interface, and vegetative cells collect in the bottom phase. Being careful not to disturb or disperse the interface layer, use a sterile 10 ml pipette to transfer the upper phase to a second sterile centrifuge tube. Discard the interface and bottom layers according to acceptable procedures for disposal of microbial waste.

j. Centrifuge this upper phase layer at 27,000 x G for 20 minutes at 5°C. Pour off the supernatant. Resuspend the spores with 20 ml sterile distilled water and pool in a sterile vessel. Pipette 25 ml aliquots of spores into sterile centrifuge tubes and wash five times with 20 ml of sterile distilled water by centrifuging (at 27,000 x G for 20 minutes at 5°C), decanting the supernatant, and re-suspending the pellet with sterile distilled water.

k. After the last wash step, resuspend each spore pellet in 20 ml filter-sterilized 50% dehydrated alcohol, USP, ethyl alcohol, 200 Proof. Pool all spore suspensions in a sterile bottle containing 15-20 sterile glass beads. Store this stock suspension at 35-40°F (2-4.4°C). The stock spore suspension may be used indefinitely if protected from evaporation and contamination.

34.A.4.3 Preparation of Spore Suspension of *Bacillus subtilis* ATCC 6633

Use the same procedure as described for *B. cereus* ATCC 11778 (Section 34.A.4.2)

34.A.4.4 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. Incubate at 35 ± 1°C for 46 to 50 hours.

To determine the dilution required to obtain 1 x 10^6 spores/ml in 50% ethyl alcohol use the following formula:

D = W/S

Where:
S = Stock concentration
W = Working concentration
D = dilution

Example:
Stock concentration = 5 x 10^8
Working concentration desired = 1 x 10^6

D = 1 x 10^6/5 x 10^8
D= 1/500

34A.5 Manual Calculation of a Standard Curve

Use the method below or the AOAC procedure Section 957.23, F for calculation.

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 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 but the average of the nine readings of the reference concentration on the set of plates is 20.3, 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. (The sum of 17.0 and '-0.3' is 16.7.)

d. Use either the method described below or the AOAC 957.23, F procedure to construct the curve.

- 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).

- Draw a line of best fit between the high and the low end points of a curve derived from the equations below.

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

  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.

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[i] Food and Drug Administration. 1968. "information for Assay and reporting of data pertaining to antibiotic residues in milk, dairy products, and animal tissue". Revised. Food and Drug Administration, Washington, D.C.