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#### A. INTRODUCTION

1. Summary of Procedure

Aminoglycosides (AMGs) residues are extracted from tissues using a buffer containing ammonium acetate / trichloroacetic acid for protein precipitation. The extract is neutralized and cleaned passing it through a weak-cation exchange sorbent using disposable pipette extraction (DPX) followed by elution with 10% formic acid in water. The final extract is analyzed using ion-pair reverse phase ultra high performance liquid chromatography (UHPLC) or hydrophillic interaction liquid chromatography (HILIC) using a high performance liquid chromatography system (HPLC) with detection by triple quadrupole mass spectrometry (MS/MS) using electrospray ionization in the positive mode (ESI<sup>+</sup>).

#### 2. Applicability

When using the UHPLC, this method is suitable for the screening and confirmation of the following aminoglycosides: amikacin, apramycin, dihydrostreptomycin, gentamicin, hygromycin B, kanamycin, neomycin B, spectinomycin (as spectinomycin hydrate), and streptomycin in bovine, poultry, and porcine kidney, bovine and porcine liver, and bovine, porcine, poultry, and equine muscle at levels found in Section J.1. in Tables11 and 12.

When using the HPLC, this method is suitable for the screening of the previously listed aminoglycosides in bovine, porcine, poultry, and equine muscle at levels found in Section J.1 Table 11.

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

#### B. EQUIPMENT

Note: Equivalent equipment may be substituted.

- 1. Apparatus
  - a. Centrifuge Thermo IEC, Centra GP-8
  - b. Cutting board and knives for mincing and removal of tendons and fat.
  - c. Vortex mixer Scientific Products, S8220
  - d. pH meter with Ag/AgCl combination electrode Orion, Model 370
  - e. Top-loading Balance Mettler, Model PB302
  - f. Analytical Balance Mettler, Model X-205 Dualrange
  - g. DPX Lever Arm Extractor DPX Labs, 24 position (Mooresville, NC)

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L.	Contrifuence turban	Delumenulano (DD) 50 ml Eolo	on Port No. 352070	
n. :		- Polypropylene (PP), 50 mL, Fait	on Part No. 352070	
i.	Centrifuge tubes -	- Polypropylene (PP), 15 mL, Faic	CON PAR NO. REF 352090	
j.	Whatman Mini-Ur 12000-524. Note: syringe filters and possible retention	hiPrep Syringless filter vials – VW Avoid glass if the Mini-UniPrep f autosampler vials, and substitute of analytes.	R 0.2 µm, PVDF, Cat. No. ilter vials are substituted with es must be checked for	
k.	Cryogenic tubes -	Nalgene, 1.2 mL, Mfr. No. 5011	0012	
l.	Nalgene FEP bot	tle – Nalgene, 30 mL, Mfr. No. 16	<b>00 00901</b>	
m.	Filters for mobile µm, Cat. No. 281	phases – VWR, Supor membrane 48-978	e disc filters, 47 mm i.d., 0.2	
n.	Sorbent Tips - DF	PX Technologies, DPX 50 mg WC	X in 5 mL tip	
Ο.	Magnetic stirrer -	Cat. No. PC-351, Corning		
p.	Repeating pipettes and tips - 25 uL- Eppendorf, 100 uL- Gilson, 1000 uL-VWR, 200 uL-Gilson, and 10 mL-Rainin.			
q.	Shaker- Eberbach Cat. No. 6010			
r.	Glassware-class A			
S.	Fluted Filter Paper - Fisher Scientific, Coarse porosity, Fast flow rate, 12.5 cm, Cat. No. 09-790-14D.			
t.	Food processor - Robot Coupe USA Inc., Robot Coupe model RSI6Y-1.			
u.	Freezer capable of -10 °C - Fisher Scientific, Isotemp Freezer, Cat. No.13-986- 149.			
<b>V</b> .	Freezer capable of -70 °C - Fisher Scientific, Isotemp Freezer Ultra-Low Temperature, Cat. No. 13-990-14.			
• W.	PVDF filter disk,	0.2 µm - Xpertek, Mfr. No. 94740	51	
<b>X</b> .	Syringe with Lue	r-Lok tip, 10 mL - Becton Dicken	son, Mfr. No. 309604	
2. Instrumentation				
а.	Waters UHPLC-I	MS/MS TQD system with MassLy	nx operating software.	
b.	UHPLC column - Pre-column UHP	- Waters UHPLC BEH C18, 2.1 x PLC BEH C18, 2.1 x 5.0 mm, 1.7	50 mm, 1.7 μm with VanGuard μm.	
<b>C.</b>	Varian 325 MS ti	riple quadruple with MS Workstati	ion operating software.	
d.	Varian 212-LC.			
е.	Varian 460-LC A	uto sampler.		
f.	Varian ProStar 500 column valve module.			

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g. Column - ZIC-HILIC (5 μm, 200 Å) 100 x 2.1 mm PEEK HPLC Column -Sequant; ZIC-HILIC (5 μm) 20 x 2.1 mm PEEK Guard column - Sequent

### C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever is soonest.

#### 1. Reagents

- a. Methanol (MeOH) LC grade, Burdick and Jackson, Cat. No. H448-10.
- b. Acetonitrile (MeCN) LC grade, CHROMASOLV, 99.9%, Sigma-Aldrich, Cat. No. HP 412.
- c. Water (H<sub>2</sub>O), LC grade house deionized water passed through Barnstead Epure 4 cartridge system.
- d. Heptafluorobutyric Acid (HFBA) Sigma, Cat. No. 77249.
- e. Hydrochloric Acid (HCI), concentrated Mallinkrodt, Cat. No. 2612.
- f. Trichloroacetic Acid (TCA) Sigma, Cat. No. T6399.
- g. Ethylenediaminetetraacetic acid, disodium salt, dihydrate (Na<sub>2</sub>EDTA•2H<sub>2</sub>O), 99+% Sigma, Cat. No. E5134.
- h. Ammonium Acetate (NH<sub>4</sub>OAc) Mallinkrodt, Cat. No. 3272.
- i. Sodium Hydroxide (NaOH) Sigma, Cat. No. S-0899.
- j. Sodium Chloride (NaCl) Sigma, Cat. No. S7653.
- k. Formic Acid (FA) Fluka, Cat. No. 94318.

#### 2. Solutions

a. 1 N HCI:

Dilute concentrated HCl 1:12 with LC water (e.g. add 7 mL acid to 77 mL water in a 100 mL glass bottle for storage).

b. 30% w/v NaOH:

Add 30 g NaOH to a 100 mL graduated cylinder containing 90 mL of LC water. Mix with a magnetic stirbar then remove with retriever.

Caution: This is an exothermic reaction; let the solution cool before adjusting to the 100 mL final volume. Store in a plastic container.

c. 1 N NaOH:

Add 4 g NaOH to a 100 mL graduated cylinder containing 95 mL of LC water. Mix with a magnetic stirbar then remove with retriever.

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Caution: This is an exothermic reaction; let solution cool before adjusting to the 100 mL final volume. Store in a plastic container.

d. 100 mM HFBA in water:

Measure 6.5 mL HFBA using a 10 mL graduated cylinder and dilute to 500 mL with LC grade water using a 500 mL volumetric flask. Store in an amber glass bottle.

e. 100 mM HFBA in MeCN:

Measure 6.5 mL HFBA using a 10 mL graduated cylinder and dilute to 500 mL with LC grade MeCN using a 500 mL volumetric flask. Store in an amber glass bottle.

f. 20 mM HFBA in water:

Measure 100 mL of 100 mM HFBA in water using a 100 mL graduated cylinder and dilute to 500 mL with LC grade water using a 500 mL volumetric flask.

g. Mobile Phase B (20 mM HFBA in MeCN):

Measure 100 mL of 100 mM HFBA in MeCN using a 100 mL graduated cylinder and dilute to 500 mL with LC grade MeCN using a 500 mL volumetric flask. Filter through a 0.2 µm membrane filter disc if necessary and transfer into UHPLC Reservoir B.

h. Mobile Phase A (20 mM HFBA in 95/5 water/MeCN):

Replace 25 mL of solution prepared in 2.f. (20 mM HFBA in water) with 25 mL of solution prepared in 2.g. (20 mM HFBA in MeCN). Filter through 0.2 µm filter disc if necessary and transfer to UHPLC Reservoir A.

Extraction solvent mixture (10 mM NH₄OAc, 0.4 mM EDTA, 1% NaCl and 2% TCA in water):

Add 1.54 g of NH<sub>4</sub>OAc to a 2 L graduated cylinder. Dilute to 1.95 L with LC water and adjust the pH to 4.0 with 1 N HCl and/or 1 N NaOH using a calibrated pH meter to measure. Add 0.3 g Na<sub>2</sub>EDTA•2H<sub>2</sub>O, 10 g of NaCl, and 40 g TCA. Mix to ensure salts dissolve and adjust final volume to 2 L with LC water. Store in >2 L glass bottle.

j. 10% FA in water:

i.

Add 10 mL of FA to a 100 mL volumetric flask containing 80 mL LC water, then fill to mark with LC water.

k. HPLC Mobile Phase (0.6% FA in water):

Add 12 mL of FA to a 2 L graduated cylinder containing 1.9 L LC water, then fill to the 2 L mark with LC water.

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### D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever ends sooner.

1. Standard Information

AMG Standards:

Amikacin, Sigma (A-1774) Apramycin HCI, Sigma (A-2024) Dihydrostreptomycin Sulfate, USP (1203008) Gentamicin Sulfate, Sigma, (G-3632) Streptomycin Sulfate, USP (1623003) Hygromycin B, Sigma, (H-7772)

Kanamycin Sulfate, Sigma (K-1876)
Neomycin B Sulfate, Sigma (N-1876)
Spectinomycin HCl, USP (1618003)
Tobramycin (int. std.), Sigma, (T-4014)

#### 2. Preparation of Standard Solution(s)

a. Individual AMG Stock solutions (2000 µg/mL in water):

For each stock solution, calculate the amount of material that contains 20 mg AMG base, accounting for purity and/or water and sulfate content. Weigh this amount to the nearest 0.1 mg. Transfer to a 30 mL Nalgene FEP bottle and add by weight (1 g/mL density for water) the exact amount of water ( $\approx$ 10 mL) to yield 2000 µg/mL concentration of the pure drug. Mix well. This standard is stable for 3 months when stored at < -10 °C.

b. Intermediate standard mixture of AMGs in water (50 µg/mL):

Pipet 250  $\mu$ L each of amikacin, apramycin, gentamicin, hygromycin B, and kanamycin, into a 30 mL FEP bottle. Add 8.75 mL of water. Mix well. This standard is stable for 3 months when stored at < -10 °C.

c. Intermediate individual standard solutions (50 µg/mL):

Pipet 250  $\mu$ L each of amikacin, apramycin, gentamicin, hygromycin B, spectinomycin, and kanamycin, into separate 30 mL FEP bottles. Add 9.75 mL of water to each bottle. Mix well. This standard is stable for 3 months when stored at < -10 °C.

d. Mixed AMG calibration/spiking solutions in water:

Following Table 1, combine the amounts of 2,000 µg/mL AMG stock solution for streptomycin, dihydrostreptomycin, neomycin and spectinomycin and for Intermediate standard mixture of AMGs in water (50 µg/mL) to prepare the mixed working standards in a 30 mL FEP bottle for kidney, liver, or muscle (use given

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volumes depending on matrix):

		1/2X	1X	2X	4X
AMG	Matrix	(mL)	(mL)	(mL)	(mL)
	Kidney	0.36	0.72	1.44	2.88
Neomycin	Liver	0.18	0.36	0.72	1.44
•	Muscle	0.06	0.12	0.24	0.48
Spectinomvcin(as	Kidney*	0.2	0.4	0.8	1.6
Spectinomycin	Liver	0.0125	0.025	0.05	0.10
Hydrate)	Muscle	0.0125	0.025	0.05	0.10
	Kidney	0.1	0.2	0.4	0.8
Streptomycin	Liver	0.025	0.05	0.1	0.2
	Muscle	0.025	0.05	0.1	0.2
· · · · · · · · · · · · · · · · · · ·	Kidney	0.1	0.2	0.4	0.8
Dihydrostreptomycin	Liver	0.025	0.05	0.1	0.2
	Muscle	0.025	0.05	0.1	0.2
Hygromycin B Amikacin Kanamycin Apramycin Gentamicin	All Matrices	0.2	0.4	0.8	1.6

Table 1 AMG Calibration/Spiking Solutions Preparation

\*Note-When preparing standards for kidney matrix, use the 50 µg/mL intermediate individual standard solution. When preparing standards for liver or muscle matrices, use the 2,000 µg/mL stock solution.

Note: The "1/2 X Recovery" sample type represents the minimum level of applicability (refer to Section J.1), with the exception of Neomycin, where 1/2X equals level of interest.

Add additional water to make 10 mL total for each solution depending on matrix:

 Table 2 Volume of Water needed to Prepare AMG Calibration/Spiking Solutions

 1/2X
 1X
 2X
 4X

 Matrix
 (ml)
 (ml)

Matrix	(mL)	(mL)	(mL)	(mL)
Kidney	9.04	8.08	6.16	2.32
Liver	9.56	9,12	8.23	6.46
Muscle	9.68	9.36	8.71	7.42

Mix well. This standard is stable for 3 months when stored at < -10  $^{\circ}$ C.

The calibration/spiking solutions yield the concentrations listed in Table 3 and are used for spiking solutions and for preparation of calibration standards (matrix-matched, screening recoveries, and external standards, if needed).

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## Table 3 AMG Calibration/Spiking Solution Concentrations in Solution

AMG	Matrix	1/2X (µg/mL)	1X (µg/mL)	2X (µg/mL)	4Χ (μg/mL)
	Kidney	72	144	288	576
Neomycin	Liver	36	72	144	288
	Muscle	12	24	48	96
Spectinomvcin(as	Kidney	1	2	4	8
Spectinomycin	Liver	2.5	5	10	20
Hydrate)	Muscle	2.5	5	10	20
	Kidney	20	40	80	160
Streptomycin	Liver	5	10	20	40
	Muscle	5	10	20	40
	Kidney	20	40	80	160
Dihydrostreptomycin	Liver	5	10	20	40
	Muscle	5	10	20	40
Hygromycin B Amikacin Kanamycin Apramycin Gentamicin	All Matrices	1	2	4	8

Table 4 gives the AMG concentrations in the matrices for screening recovery samples when 100  $\mu L$  is spiked into 2 g samples and 25  $\mu L$  is added to 0.5 g equivalent final extracts:

AMG	Matrix	1/2X (μg/g)	1X (µg/g)	2X (µg/g)	4X (µg/g)
	Kidnev	3.6	7.2	14.4	28.8
Neomycin	Liver	1.8	3.6	7.2	14.4
	Muscle	0.6	1.2	2.4	4.8
Spectinomycin (as	Kidney	0.05	0.1	0.2	0.4
Spectinomycin	Liver	0.125	0.25	0.5	1
Hvdrate)	Muscle	0.125	0.25	0.5	1
	Kidney	1	2	4	8
Streptomycin	Liver	0.25	0.5	1	2
	Muscle	0.25	0.5	1	2
	Kidney	1	2	4	8
Dihvdrostreptomycin	Liver	0.25	0.5	1	2
	Muscle	0.25	0.5	1	2
Hygromycin B Amikacin Kanamycin Apramycin Gentamicin	All Matrices	0.05	0.1	0.2	0.4

Table 4 AMG Screening Calibration/Spiking Solutions/Concentrations in Matrix

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Table 5 provides individual stock standard and individual intermediate standard volumes (in mL) needed to prepare confirmation recovery spiking solutions to be used for each specific species and tissue analysis:

Table 5 Volume of Stock or Intermediate Standards Needed per Analyte for Confirmation Analyses

AMG	Bovine and Porcine Kidney (mL)	Bovine Liver (mL)	Porcine Liver (mL)	Bovine Muscle (mL)	Porcine Muscle (mL)	Poultry Kidney (mL)	Pouitry Muscle (mL)	Equine Muscie (mL)
Amikacin*	0.2	0.4	0.2	0.2	0.2	0.2	0.2	0.2
Apramycin*	0.2	0.8	0.2	0.4	0.2	0.2	0.2	0.2
Dihydrostreptomycin	0.1	0.1	0.025	0.025	0.025	0.1	0.025	0.025
Gentamicin*	0.4	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Hygromycin B*	0.2	0.2	0.2	0.8	0.2	0.2	0.2	0.2
Kanamycin*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Neomycin	0.36	0.18	0.18	0.12	0.06	0.36	0.06	0.06
Spectinomycin* (as Spectinomycin Hydrate)	0.2	1	0.5	0.5	1	0.4	0.5	2
Streptomycin	0.1	0.1	0.025	0.025	0.025	0.1	0.025	0.025
Water (mL)	8.04	6.22	7.67	6.93	7.29	7.44	7.79	6.29
Total Volume (ML)	10	10	10	10	10	10	10	10

\*Note-The volumes listed for Amikacin, Apramycin, Gentamicin, Hygromycin B, Kanamycin and Spectinomycin are using the 50  $\mu$ g/mL intermediate individual standard solutions. The 2,000  $\mu$ g/mL stock solution may be used instead but appropriate changes to the volume of water added must be made.

The volumes listed for Streptomycin, Dihydrostreptomycin and Neomycin are using the 2000 µg/mL stock solution.

Table 6 provides AMG concentrations in solution for the standards prepared in Table 5. These standards are for use as spiking solutions for confirmation analyses.

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### Table 6 AMG Confirmation Calibration/Spiking Concentrations

AMG	Bovine and Porcine Kidney (µg/mL)	Bovine Liver (µg/mL)	Porcine Liver (µg/mL)	Bovine Muscle (µg/mL)	Porcine Muscle (µg/mL)	Pouitry Kidney (µg/mL)	Poultry Muscle (µg/mL)	Equine Muscle (µg/mL)
Amikacin	1	2	1	1	1	1	1	1
Apramycin	.1	4	1	2	1	1	1	1
Dihydrostreptomycin	20	20	. 5	5	5	20	5	5
Gentamicin	2	4	4	4	.4	4	4	4
Hygromycin B	1	1	1	4	1	1	1	1 .
Kanamycin	1	1	1	1	1	1	1	1
Neomycin	72	36	36	24	12	72	12	12
Spectinomycin (as Spectinomycin Hydrate)	1	5	2.5	2.5	5	2	2.5	10
Streptomycin	20	20	5	5	5	20	5	5

Table 7 provides AMG concentrations for different matrices in confirmation recovery samples using 100  $\mu$ L (of standards used from Table 6) spiked into 2 g samples:

AMG	Bovine and Porcine Kidney (ug/g)	Bovine Liver (ug/g)	Porcine Liver (ug/g)	Bovine Muscle (ua/a)	Porcine Muscle (ug/g)	Poultry Kidney (ua/a)	Poultry Muscle (ug/g)	Equine Muscle (ua/a)
Amikacin	0.05	0.10	0.05	0.05	0.05	0.05	0.05	0.05
Apramycin	0.05	0.2	0.05	0.1	0.05	0.05	0.05	0.05
Dihydrostreptomycin	1	1	0.25	0.25	0.25	1	0.25	0.25
Gentamicin	0.1	0.2	0.2.	0.2	0.2	0.2	0.2	0.2
Hygromycin B	0.05	0.05	0.05	0.2	0.05	0.05	0.05	0.05
Kanamycin	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Neomycin	3.6	1.8	1.8	1.2	0.6	3.6	0.6	0.6
Spectinomycin (as Spectinomycin Hydrate)	0.05	0.25	0.125	0.125	0.25	0.1	0.125	0.5
Streptomycin	1	1	0.25	0.25	0.25	1	0.25	0.25

#### Table 7 AMG Confirmation Calibration/Spiking Concentrations in Matrix

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e. Tobramycin Internal Standard (IS) in water (40 µg/mL):

Pipet 200  $\mu$ L of 2000  $\mu$ g/mL tobramycin stock solution from D.2.a into 9.8 mL of water in a 30 mL FEP bottle. Mix well. This standard is stable for 3 months when stored at < -10 °C.

f. AMG External Standard in 9% FA Solution:

Add 25  $\mu$ L of appropriate calibration/spiking solutions (see standards/concentrations in Table 3) plus 25  $\mu$ L of 40  $\mu$ g/mL IS solution to the labeled bottom portions of Whatman Mini-Uniprep autosampler vials. Add 0.45 mL of 10% FA in water. Filter the reagent-only calibration standard by placing the upper filter caps on the bottom portion of the vials. Mix well by vortexing. Inject the 1/2 X standard prior to each day's run of samples to determine the instrument's suitability. These solutions can be stored at 2 - 8 °C and re-used for five days for routine monitoring.

g. Matrix-Matched Calibration Standard

Refer to Section F for preparation of final extracts. Add 25  $\mu$ L of appropriate calibration/spiking solutions (see standards/concentrations in Table 3) plus 25  $\mu$ L of 40  $\mu$ g/mL IS solution to the labeled bottom portions of Whatman Mini-Uniprep autosampler vials. Add 0.45 mL of blank final extracts for the appropriate matrix. Filter the matrix-matched calibration standard(s) (1/2X, X, 2X and 4X) by placing the upper filter caps on the bottom portion of the vials. Mix well by vortexing. For the matrix blank or negative control (0X), add 25  $\mu$ L of water rather than a calibration/spiking solution. These solutions can be stored at 2 - 8°C and re-used for five days for routine monitoring.

Note: Thorough mixing is critical for the preparation of these standards

Note: Solutions are stable for three months when stored at <-10 °C, five days when stored at 2 - 8 °C, and one day at ambient temperatures.

#### E. SAMPLE PREPARATION

Samples collected fresh must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (< -10 °C) prior to mincing/grinding if they cannot be prepared on the day of receipt. If sample is frozen, allow to thaw, but keep as cold as possible. Dissect away fat and connective tissue from kidney or liver. Mince finely or grind tissue in blender or vertical cutter-mixer. Store samples frozen (<-10 °C) prior to analysis.

Sample preparation may also be done by dry ice grinding as follows;

- a. Chop 0.5 -1 lb of muscle tissue into small pieces and homogenize with an equal amount of dry ice in a large food processor. The resulting sample homogenate will be a frozen powder.
- b. Transfer a portion of the homogenized sample into a loosely capped sample cup

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until the dry ice has sublimed. Excess sample from step E.a may be discarded.

c. For any retained sample, tighten the caps and store sample cups at  $\leq$  -10 °C.

#### F. ANALYTICAL PROCEDURE

- 1. Preparation of Controls and Samples
  - a. Weigh 2.0 ± 0.1 g of known blank tissue into a 50 mL polypropylene centrifuge tube, allow tissues to thaw and do the following described below: Note: Use corresponding blank tissue for controls for each specific species and tissue sample being analyzed.
    - i. Screening Prepare one each for a blank (negative control), a recovery (positive control), a matrix matched standard, and a check sample, if necessary. For bovine liver an additional 2 g portion of blank tissue will be needed.
    - ii. Confirmation Weigh four 2 g portion(s) of blank tissue into 50 ml polypropylene centrifuge tubes. One for recovery (positive controls), and three for the blank and the matrix matched standards. Weigh additional portion for check sample, if necessary.
    - iii. Prepare recovery at this time by fortifying one of the blank tissue portions with 100  $\mu$ L of the appropriate calibration/spiking solutions (1/2 X for screening or the confirmation spiking solution). For bovine liver, an additional blank tissue portion will be spiked with 100  $\mu$ L of the 1 X calibration/spiking solution to serve as the 1 X recovery (positive control).
- 2. Extraction Procedure
  - a. Weigh 2.0 ± 0.1 g of tissue into a 50 mL polypropylene centrifuge tube, allow tissue to thaw.
  - b. Add 20 mL of NH<sub>4</sub>OAc/EDTA/NaCI/TCA buffer to each tube.
  - c. Add 100 µL of the 40 µg/mL tobramycin IS to yield 2 µg/g in the tissue.

Note: Do <u>not</u> fortify the blank tissue portion used to create matrix-matched standards with IS.

- d. Shake for 10 minutes.
- e. Centrifuge at approximately 3700 rcf for three minutes. If floating material is observed, remove it with a spatula.
- f. Decant >10 mL supernatant into another labeled 50 mL PP tube. If dry ice grinding was done for sample preparation, decant the supernatant through a fluted paper filter.
- g. Using a calibrated pH meter, adjust pH of the sample extracts to 6.50 ± 0.05 with

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a few drops of 30% NaOH followed by 1 N NaOH and/or 1 N HCl for fine adjustment.

- h. Centrifuge at approximately 3700 rcf for three minutes.
- i. Pipet 10 mL of extract into a labeled 15 mL PP tube (this is 1 g sample equivalent when using the IS).

Note: Extracts may be filtered using a 0.2 µm PVDF filter disk and 10 mL syringe as needed prior to DPX cleanup.

j. For DPX cleanup place the WCX-DPX tips in the lever arm apparatus, including the rubber O-ring to ensure gas tight seal. Position the lever arm to give 3 mL of air in the plastic syringes, and then attach the manifold cover.

- k. Place the following tubes per sample extract from step i. for cleanup:
  - i. 15 mL PP tubes with 3 mL of MeOH for conditioning step l.i.
  - ii. 15 mL PP tubes with 3 mL LC water for conditioning step I.ii.
  - iii. 15 mL PP tubes from step i. with 10 mL extracts to be loaded onto sorbent in tips per step m.
  - iv. 15 mL empty PP tubes for waste solvent from sample loading step m.
  - v. 15 mL PP tubes with 5 mL of LC water for sorbent rinse step n.
  - vi. 15 mL PP tubes with 0.9 mL of 10% FA in water for analyte elution step o.
- I. Condition the 50 mg WCX tip:

р.

- i. Pump once with 3 mL of MeOH.
- ii. Pump twice with 3 mL of LC water.
- m. Load all 10 mL of each sample onto sorbent tip in 4 x 2.5 mL portions.
  - i. Make sure that the sorbent is interacting with the extract for at least 30 seconds.
  - ii. Use two pumps for the first 2.5 mL portion of extract before discarding the extract solvent into the waste tube.
  - iii. Process the three remaining 2.5 mL portions of sample with one pump each.
- n. Wash each tip with 5 mL of LC water to rinse away possible contaminants.
- o. Elute the sorbent with 900 µL of 10% formic acid in water.
  - i. Load 900 µL into the tip and pump five times.
  - ii. Always let the sorbent interact with the solvent for 30 seconds.
  - Add 100 µL of water to each cleaned extract and mix well. Do not add the 100 µL to the blank extract(s) that will be used to prepare the matrix-matched

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#### standard(s).

- Screening For the matrix matched standard, combine 0.45 mL of eluant and calibration standards as described in step D.2.g.
- Confirmation For the matrix blanks, combine the three matrix blanks into one of the 15 mL tubes (2.7 mL total volume). As described in step D.2.g, 0.45 mL is used for the negative control (blank) and the four matrixmatched calibration standards.
- q. For the samples and spikes, place 500 µL of each final extract into bottom piece of Mini Uni-Prep PVDF syringeless filter vial. Then insert top filter vial and press together.

#### 3. Instrumental Settings

Í.

Note: The instrument parameters may be optimized to ensure system suitability.

#### **UHPLC-MS-MS analysis**

Instrument Operating Parameters – UHPLC system

a. Mobile phases for AMG analysis:

Mobile Phase A – 95% water / 5% MeCN / 20 mM HFBA Mobile Phase B – 100% MeCN / 20 mM HFBA

Flush column with 1:1 A/B at a flow rate of 0.5 mL/min for three minutes. Change the mobile phase initial conditions to 100% A. Allow column to equilibrate until the "delta" value on the pressure reading is < 20.

b. UHPLC gradient program: (Table 8)

Flow rate: 0.5 mL/min Pressure Limits: 200 psi minimum; 12,000 psi maximum Run time: 3.00 min

Table 8 – LC gradient

	X		
Time (min)	% Mobile Phase A	% Mobile Phase B	Gradient
0.00	100	0	none
0.50	80	20	linear
1.00	80	20	none
2,00	60	40	linear
2.05	10	2 <b>90</b>	linear
2.50	10	90	none
2.55	100	0	linear
3.00	100	0	none

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~	Autos	ompler or	oaram		
0.	i	Dun tim	e: 3.0 min		
				)	
	11. 131		tion: Dartial loop with needle overfi	11	
	10. 5 <i>7</i>	Loop of			
	IV.	Mooku	voob colvent: Mobile Phase A		
	V	Weak w			
	VI.	Strong	vash solvort: Mobile Phase B		
	VII.	Strong	wash solvent. Mobile Flase D		
	VIII.	Strong	temperature: 7°C		
	IX.	Sample			
	х.	Column	Manager.	Jump location	
		(a) (b)	Column monogor tomporature: 40		
		(D)	Les divert volve to divert eluant to	upste 0.25 minutes prior to first	
		(C)	peak and 0.25 minutes after last an	alyte peak.	
d.	Instru	ment Ope	erating Parameters – Mass Spectro	meter	
	i.	Mass S manufa solutior	pectrometer calibration and resoluti cturer's specification using the man n.	ion were done according to the ufacturer's supplied calibration	
	ii.	Type: 1	MS/MS		
	111.	Electro	spray Source Parameters: Capillary (kV): 3.0 Cone (V): Variable - analyte deper Extractor (V): 3.0 RF (V): 0.10 Source Temperature (°C): 150 Desolvation Temperature (°C): 450 Cone Gas Flow (L/hr): 20 Desolvation Gas Flow (L/hr): 900 Collision Gas Flow (mL/min): 0.20	ndent D	
	iv.	Analyz	er Parameters: LM 1 Resolution: 12.50 HM 1 Resolution: 12.50 MSMS Mode Entrance: -5 MSMS Mode Collision Energy: Var MSMS Mode Exit: 1	iable – analyte dependent	

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LM 2 Resolution: 12.50 HM 2 Resolution: 12.50						
v. MS Method Parameters: Type: MRM Ion Mode: ES+ Dwell (s): 0.005 Start time (min): 0.8 End time (min): 2.6						
Start-End	Dwell Time		Precursor	Product	Cone	Collision
Time (min)	(ms)	Compound	ion (m/z)	ions (m/z)	(V)	Energy (V)
0.9-1.2	22	Spectinomycin Hydrate	351.24	<b>98.00</b> 333.33 140.10	40	30 20 25
1.1-1.3	22	Hygromycin B	528.20	<b>177.05</b> 352.03 257.00	44	30 24 30
1.2-1.4	22	Streptomycin	582.17	263.09 246.05 176.00	70	32 40 40
1.2-1.4	52	Dihydrostreptomycin	584.17	263.09 246.05 176.00	70	30 38 40
1.5-1.7	50	Amikacin	586.43	<b>163.21</b> 247.37 101.98	35	35 45 40
1.6-1.8	50	Kanamycin A	485.36	<b>163.22</b> 324.33 102.14	. 30	20 15 45
1.9-2:1	11	Apramycin	540.41	<b>217.20</b> 378.31 199.35	35	25 15 35
2.0-2.1	11	Tobramycin (IS)	468.36	<b>163.19</b> 145.10	25	25 30
2.0-2.2	11	Gentamicin c1a	450.39	<b>160.16</b> 322.37 112.17	35	25 15 30
2.0-2.2	11	Gentamicin c2+c2a	464.42	<b>160.23</b> 322.39 163.14	35	25 15 20
2.0-2.3	11	Gentamicin c1	478.42	<b>157.25</b> 160.16 322.42	40	30 25 15
2.1-2.3	11	Neomycin B	615.30	<b>163.38</b> 293.03 160.53	52	35 24 33

Note: Product ions are listed with the screening/confirmation ion in bold (top) followed by diagnostic ions 1 and 2 (middle and bottom) for screening/confirmation purposes of each analyte. Ratios are calculated by

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dividing the amount of each diagnostic ions to the confirmation ion. The ions used and relation may vary with other analytical systems. Ion ratios should be less than 1. If this ratio is not less than one for a sample set, the inverse of this ratio may be used.

See Appendix for diagrams of proposed fragmentation patterns for each analyte.

Note: Confirmation of the presence of spectinomycin hydrate is considered to be confirmation of the parent spectinomycin.

- e. UHPLC-MS/MS Analytical Procedure
  - i. Turn on UHPLC pump, set mobile phase to 100% A at a flow rate of 0.50 mL/min. Perform column equilibration for five minutes. Verify backpressure of column gives "delta" value < 20 in pressure fluctuations.
  - ii. Turn on MS and load appropriate MS Tune file (.ipr). Turn on API gas flow. Allow MS to achieve designated gas flow and desolvation temperature. Place MS valve position to LC.
  - iii. Inject 15 µL of external standard (appropriate for the tissue to be analyzed), followed by two injections of 10% FA in water (solvent blank).
     Verify the retention time, divert valve switching time, and detection of MS/MS ions using the TargetLynx sample processing program.
  - iv. Inject matrix-matched standard(s). Then inject recovery(ies), blank, followed by samples. One may want to put solvent blanks in between samples in case of high finding leads to carry-over.
  - v. As a test of retention time and instrument response stability, re-inject a calibration standard at the end of the injection sequence. Depending on instrument variability, additional injection of control standards may be interspersed mid-sample sequence.
  - vi. Column, Pump and ES interface care: At the end of set of analyses, the column should be flushed for 5-10 minutes with Acetonitrile. Then the instrument performs a shutdown procedure, turning off LC flow and MS desolvation temperature and gas flow. Inspect entrance cone for cleaning, following manufacturer's specification for cleaning the surfaces.
- 4. HPLC-MS-MS Instrumental Settings (Muscle Tissue Screening Only)

Note: The instrumental parameters may be optimized to ensure system suitability.

- a. Mobile phase for AMG analysis:
  - 0.6% FA in water.
- b. HPLC settings:

Flow rate: 0.6 mL/min

Pressure Limits: 200 psi minimum; 3,000 psi maximum

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	Pun time: 15.0 min	
	Gradient: isocratic	
•	Auto sampler program:	
U.		
	Needle tubing volume: 15 ul	
	Mash solvent: 0.6% formic acid	
	Number of washes: 5	
	Sample temperature: ambient	
٦		
u.	Column valve module.	n
	Mobile phase valve position: to match mobile p	hase location
	Column tomporature: 30 °C	
	Column temperature. 30 C	
•	MS Operating Parameters	
е.	MS Operating Parameters	were done according to the
e.	MS Operating Parameters Mass Spectrometer calibration and resolution v manufacturer's specification using the manufac solution.	vere done according to the turer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution v manufacturer's specification using the manufac solution. Type: MS/MS	vere done according to the turer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution v manufacturer's specification using the manufac solution. Type: MS/MS Scan Method Parameters:	vere done according to the sturer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution w manufacturer's specification using the manufact solution. Type: MS/MS Scan Method Parameters: Ionization: vESI	vere done according to the sturer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution v manufacturer's specification using the manufact solution. Type: MS/MS Scan Method Parameters: lonization: vESI Q1 peak width selection: 1.5	vere done according to the turer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution v manufacturer's specification using the manufact solution. Type: MS/MS Scan Method Parameters: lonization: vESI Q1 peak width selection: 1.5 Q3 peak width selection: 1.5	vere done according to the sturer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution w manufacturer's specification using the manufact solution. Type: MS/MS Scan Method Parameters: lonization: vESI Q1 peak width selection: 1.5 Q3 peak width selection: 1.5 CID gas pressure: 2.40	vere done according to the sturer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution w manufacturer's specification using the manufact solution. Type: MS/MS Scan Method Parameters: lonization: vESI Q1 peak width selection: 1.5 Q3 peak width selection: 1.5 CID gas pressure: 2.40 Needle voltage positive: 6.0 kV	vere done according to the sturer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution w manufacturer's specification using the manufact solution. Type: MS/MS Scan Method Parameters: lonization: vESI Q1 peak width selection: 1.5 Q3 peak width selection: 1.5 CID gas pressure: 2.40 Needle voltage positive: 6.0 kV Drying gas temperature: 350 °C	vere done according to the cturer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution w manufacturer's specification using the manufact solution. Type: MS/MS Scan Method Parameters: Ionization: vESI Q1 peak width selection: 1.5 Q3 peak width selection: 1.5 CID gas pressure: 2.40 Needle voltage positive: 6.0 kV Drying gas temperature: 350 °C Nebulizer gas pressure: 70.0 psi	vere done according to the sturer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution w manufacturer's specification using the manufacture solution. Type: MS/MS Scan Method Parameters: Ionization: vESI Q1 peak width selection: 1.5 Q3 peak width selection: 1.5 CID gas pressure: 2.40 Needle voltage positive: 6.0 kV Drying gas temperature: 350 °C Nebulizer gas pressure: 70.0 psi Drying gas pressure: 45.0 psi	vere done according to the sturer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution w manufacturer's specification using the manufacturer's solution. Type: MS/MS Scan Method Parameters: Ionization: vESI Q1 peak width selection: 1.5 Q3 peak width selection: 1.5 CID gas pressure: 2.40 Needle voltage positive: 6.0 kV Drying gas temperature: 350 °C Nebulizer gas pressure: 70.0 psi Drying gas pressure: 45.0 psi Vortex gas pressure: 10.0 psi	vere done according to the cturer's supplied calibration
e.	MS Operating Parameters Mass Spectrometer calibration and resolution we manufacturer's specification using the manufact solution. Type: MS/MS Scan Method Parameters: Ionization: vESI Q1 peak width selection: 1.5 Q3 peak width selection: 1.5 CID gas pressure: 2.40 Needle voltage positive: 6.0 kV Drying gas temperature: 350 °C Nebulizer gas pressure: 70.0 psi Drying gas pressure: 45.0 psi Vortex gas pressure: 10.0 psi Vortex gas temperature: 250 °C	vere done according to the sturer's supplied calibration

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Scan width in SIM and MRM mode: 0.70 amu

Detector: use EDR

### Table 10 - MRM Transitions

	1	1			•	
Segment Start-End Time (min)	Dwell Time (s)	Compound	Precursor ion (m/z)	Product ion (m/z)	Capillary (V)	Collision Energy (V)
0.4-1.40	0.1	Spectinomycin Hydrate	351.24	98.00	84.0	28.0
0.4-1.40	0.1	Hygromycin B	528.20	177.05	92.0	27.5
0.4-1.40	0.1	Streptomycin	582.17	263.09	176.0	31.0
0.4-1.40	0.1	Dihydrostreptomycin	584.17	263.09	148.0	29.0
1.40-3.25	0.1	Amikacin	586.43	163.21	88.0	30.5
1.40-3.25	0.1	Kanamycin A	485.36	163.22	68.0	23.0
3.25-7.50	0.3	Apramycin	540.41	217.20	84.0	24.5
3.25-7.50	0.2	Tobramycin (IS)	468.36	163.19	68.0	20.5
3.25-7.50	0.3	Gentamicin c1a	450.39	160.16	52.0	22.5
3.25-7.50	0.3	Gentamicin c2+c2a	464.42	160.23	52.0	23.0
3.25-7.50	0.3	Gentamicin c1	478.42	157.25	60.0	22.5
7.50-13.0	0.1	Neomycin B	615.30	163.38	92.0	31.5

- 5. Injection sequence /Sample Set
  - a. Screening Set
    - i. External Standard (optional)
    - ii. Matrix matched standard
    - iii. Recovery(ies)
    - iv. Check sample (if necessary)

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- v. Blank
- vi. Up to 21 Samples
- vii. External standard, matrix matched standard, or positive control
- b. Confirmation Set
  - i. External Standard (optional)
  - ii. Matrix matched standards
  - iii. Recovery(ies)
  - iv. Check sample (if necessary)
  - v. Matrix Matched Blank
  - vi. Up to 20 Samples
  - vii. External standard, matrix matched standard, or positive control

### G. CALCULATIONS / IDENTIFICATION

- 1. Screening
  - a. The retention times for the recoveries must match the retention time of the matrix-matched standard within 5%. Retention time for the samples must match the retention time of the positive control or the matrix matched standard within 5%.
  - b. Blank must be less than 10% of the 1/2 X level for the matrix-matched standard.
  - c. The screening ion for a given analyte must be present. The required ion for each compound is listed in Table 9 or 10.
  - d. The screening ion must have a signal-to-noise ratio  $\geq$  3. This may be verified by visual inspection.
  - e. A sample is screened positive for an analyte if the following criteria are met:
    - i. The fortified recovery of the analyte must exceed 10% of the 1/2 X matrix matched standard level.
    - ii. The sample response equals or exceeds the 1/2 X (or level of interest) recovery standard (positive control) level.
- 2. Confirmation
  - a. Monitored ions for each AMG will be assessed as follows:
    - i. Recovery retention times must match the retention time of the matrix matched standard within 5%. Retention time for the samples must match the retention time of the positive control or the matrix matched standard within 5%.

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	ii.	All produc noise rati	ct ions specified for ratio ma $o ≥ 3$ . This may be verified	tching by visu	are present with a signal-to- al inspection.
	iii.	One of th	e following ion ratio matchin	g conc	litions is met:
		(a) If m st	two product ions are assess atch the calculated average andards within a $\pm$ 10% abs	ed, on ratio c olute d	e sample ion ratio should of the matrix-matched lifference.
	(b) If three product ions are monitored, the presence of two samp ratios should match the calculated average ratio of the matrix- matched standards within a ± 20% absolute difference.				
	iv. The fortified recovery of the analyte must exceed 10% of the one-half X matrix matched standard level.				
	v. The blank must be less than 10% of the 1/2 X level for the matrix matched standard.				
	vi. The sample response equals or exceeds the recovery standard (positive control) level.				
	Note: If a sample shows a positive response for a compound which did not meet confirmation criteria in the associated QC samples, then further testing of that sample is warranted.				
H.	SAFETY INF	ORMATIO	N AND PRECAUTIONS		
1.	Required Pro applicable.	tective Equ	uipment - Protective clothing	, eyew	rear, and gloves, where
2.	Hazards				
	Procedure Step	)	Hazard	Red	commended Safe Procedures
A	MG Standards	Stan dama	dards can cause kidney age	Wea glov	ar protective clothing and es when handling standards.
A	cetonitrile	Flam Vapo Irritat	mable. Explosive hazard. ors will explode if ignited. ting to skin and mucous	Kee awa fum	p container tightly closed and y from fire. Use under a e hood. Avoid breathing

membranes. vapors. Concentrated Acids: Corrosive substances. Danger HCI, Acetic, HFBA, of chemical burns. Potential for TCA, Formic, and inhalation of corrosive fumes. solutions. Prepare solutions in a fume hood. Wear protective equipment and avoid contact with skin.

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Procedure Step	Hazard	Recommended Safe Procedures
NaOH and solutions made from same	Corrosive substances Danger of chemical burns.	Wear gloves when preparing solutions, and take care to avoid splashes or spills.
Dry Ice	asphyxiation, cold contact burns	Use only in a well-ventilated

space and avoid reaching too far into the dry ice container. Wear the appropriate gloves while working with the dry ice.

#### 3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

### I. QUALITY ASSURANCE PLAN

### 1. Performance Standard

- a. For screening:
  - i. For set acceptance, the nine analytes in the fortified recovery (positive control) must meet screening criteria.
  - ii. The blank (negative control) must be negative using the criteria in Section G.
- b. For Confirmation:
  - i. For set acceptance, eight of the nine analytes must meet confirmation criteria. For sample reporting purposes, the analytes of interest in the fortified recovery (positive control) must meet confirmation criteria.
  - ii. The blank (negative control) must be negative using the criteria in Section G for the analytes of interest.
- 2. Critical Control Points and Specifications

#### Record

Acceptable Control

a. None known

- 3. Intralaboratory Check Samples
  - a. System, minimum contents.
    - i. Frequency: One per week per analyst when samples analyzed.

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- ii. Records are to be maintained.
- b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

- i. Investigate following established procedures.
- ii. Take corrective action as warranted.
- 4. Condition upon receipt: cool or frozen.

### J. APPENDIX

- 1. Screening and Confirmation Levels
  - a. Screening

Table 11 - Minimum Level of Applicability per Species

AMG	Matrix	Bovine (µg/g)	Porcine (µg/g)	Poultry (µg/g)	Equine (µg/g)
	Kidney	0.05	0.05	0.05	N/App
Amikacin	Liver	0.1	0.05	N/App	N/App
	Muscle	0.05	0.05	0.05	0.05
	Kidney	0.05	0.05	0.05	N/App
Apramycin	Liver	0.1	0.05	N/App	N/App
· •	Muscle	0.05	0.05	0.05	0.05
· · · · · · · · · · · · · · · · · · ·	Kidney	1	1	1	N/App
Dihydrostreptomycin	Liver	0.5	0.25	N/App	N/App
	Muscle	0.25	0.25	0.25	0.25
	Kidney	0.05	0.05	0.05	N/App
Gentamicin	Liver	0.1	0.05	N/App	N/App
	Muscle	0.05	0.05	0.05	0.05
	Kidney	0.05	0.05	0.05	N/App
Hygromycin B	Liver	0.1	0.05	N/App	N/App
	Muscle	0.05	0.05	0.05	0.05
	Kidney	0.05	0.05	0.05	N/App
Kanamycin	Liver	0.1	0.05	N/App	N/App
•	Muscle	0.05	0.05	0.05	0.05

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AMG	Matrix	Bovine (µg/g)	Porcine (µg/g)	Poultry (µg/g)	Equine (µg/g)
	Kidney	0.45	0.45	0.45	N/App
Neomycin*	Liver	1.8	1.8	N/App	N/App
-	Muscle	0.6	0.6	0.6	0.6
	Kidney	0.05	0.05	0.05	N/App
Spectinomycin	Liver	0.125	0.125	N/App	N/App
. ,	Muscle	0.125	0.125	0.125	0.125
	Kidney	1	1	1	N/App
Streptomycin	Liver	0.5	0.25	N/App	N/App
	Muscle	0.25	0.25	0.25	0.25

\*Note- Neomycin screening and confirmation minimum applicable levels are lower than the screening and confirmation levels as described in the standard tables in section D. Changes to these levels may occur in association with 21CFR requirements as updated by FDA.

b. Confirmation

Table 12 - Minimum Level of Applicability per Species

AMG	Matrix	Bovine (µa/a)	Porcine (µg/g)	Poultry (µg/g)	Equine (µg/g)
	Kidney	0.05	0.05	0.05	N/App
Amikacin	Liver	0.1	0.05	N/App	N/App
	Muscle	0.05	0.05	0.05	0.05
	Kidney	0.05	0.05	0.05	N/App
Apramycin	Liver	0.2	0.05	N/App	N/App
• •	Muscle	0.1	0.05	0.05	0.05
	Kidney	1	1	1	N/App
Dihydrostreptomycin	Liver	1	0.25	N/App	N/App
	Muscle	0.25	0.25	0.25	0.25
	Kidney	0.1	N/App	0.2	N/App
Gentamicin	Liver	N/App	0.2	N/App	N/App
	Muscle	N/App	0.2	0.2	0.2
	Kidney	0.05	0.05	0.05	N/App
Hygromycin B	Liver	N/App	0.05	N/App	N/App
	Muscle	0.2	0.05	0.05	0.05
· · · · · · · · · · · · · · · · · · ·	Kidney	0.05	0.05	0.05	N/App
Kanamycin	Liver	N/App	0.05	N/App	N/App
-	Muscle	0.05	0.05	0.05	0.05

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AMG	Matrix	Bovine (µg/g)	Porcine (µg/g)	Poultry (µg/g)	Equine (µg/g)
<u> </u>	Kidney	0.45	0.45	0.45	N/App
	Liver	N/App	1.8	N/App	N/App
Neomycin	Muscle	1.2	0.6	0.6	0.6
	Kidney	0.05	0.05	0.1	N/App
Spectinomycin	Liver	0.25	0.125	N/App	N/App
• •	Muscle	0.125	0.25	0.125	0.5
· · · · · · · · · · · · · · · · · · ·	Kidnev	1	1	1	N/App
Streptomycin	Liver	1	0.25	N/App	N/App
	Muscle	0.25	0.25	0.25	0.25

N/App = Not Applicable

\*Note- Neomycin screening and confirmation minimum applicable levels are lower than the screening and confirmation levels as described in the standard tables in section D. Changes to these levels may occur in association with 21CFR requirements as updated by FDA.

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#### 2. Chromatograms/spectra

UHPLC a.





1.880 2000

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100

9

0

100-

•%

n

1007

\*

0

FIONRM

F10:MPM





🗯 min

min

2 200 min

3 channels,69+ 478 42+180.18 1 208#+003

F12 eRM of 3 channels,E8+ 478 42+322 42 8 611 e+002

Geniamicin c 1

100 \*

۵

F12:M 100 -

0

100 -

0

2 000







F13:MFM of 3 channels,E8+ 01630-160 53 100 2.16 2.16 2.16 8 215 239 42 8 1555 2.200 min 0.1 2.000



Centamicin c2 +c2s

## AR0002066

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### b. HPLC



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### 3. Proposed fragmentation patterns

Amikacin

Formula:  $C_{22}H_{43}N_50_{13}$  MW: 585.60 g/mol m/z 586.43  $\rightarrow$  m/z 247.37 m/z 586.43  $\rightarrow$  m/z 163.21 m/z 586.43  $\rightarrow$  m/z 101.98

юн m/z 247 но но OF m/z 163 m/z 686

m/z 102

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Apramycin

Formula:  $C_{21}H_{41}N_5O_{11}$  MW: 539.28 g/mol m/z 540.41  $\rightarrow$  m/z 378.31 m/z 540.41  $\rightarrow$  m/z 217.20 m/z 540.41  $\rightarrow$  m/z 199.35



Dihydrostreptomycin

MW: 583.21 g/mol

mycin Formula:  $C_{21}H_{41}N_7O_{12}$  *m/z* 584.17 → *m/z* 263.09 *m/z* 584.17 → *m/z* 246.05 *m/z* 584.17 → *m/z* 176.00



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Gentamicin C<sub>1</sub>

MW: 477.32 g/mol

 $m/z 478.42 \rightarrow m/z 322.42$  $m/z 478.42 \rightarrow m/z 160.16$ 

Formula: C21H43N507

m/z 478.42  $\rightarrow m/z$  157.25



Gentamycin C<sub>1a</sub>

Formula:  $C_{19}H_{39}N_5O_7$  MW: 449.29 g/mol m/z 450.39  $\rightarrow$  m/z 322.37 m/z 450.39  $\rightarrow$  m/z 160.16 m/z 450.39  $\rightarrow$  m/z 112.17



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Formula: C<sub>20</sub>H<sub>41</sub>N<sub>5</sub>O<sub>7</sub>

Gentamicin C<sub>2</sub> + C<sub>2a</sub>

MW: 463.30 g/mol

m/z 464.42  $\rightarrow m/z$  322.39 m/z 464.42  $\rightarrow m/z$  163.14

m/z 464.42  $\rightarrow m/z$  160.23



Hygromycin B

Formula:  $C_{20}H_{37}N_{3}O_{13}$  MW: 527.23 g/mol m/z 528.20  $\rightarrow m/z$  352.03 m/z 528.20  $\rightarrow m/z$  257.00 m/z 528.20  $\rightarrow m/z$  177.05



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Kanamycin A

MW: 484.24 g/mol

 $m/z 485.36 \rightarrow m/z 324.33$  $m/z 485.36 \rightarrow m/z 163.22$  $m/z 485.36 \rightarrow m/z 102.14$ 

Formula: C<sub>18</sub>H<sub>3</sub>N<sub>4</sub>O<sub>11</sub>



**Neomycin B** 

Formula:  $C_{23}H_{46}N_6O_{13}$  MW: 614.31 g/mol m/z 615.30  $\rightarrow$  m/z 293.03 m/z 615.30  $\rightarrow$  m/z 163.38 m/z 615.30  $\rightarrow$  m/z 160.53



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Spectinomycin hydrate

Formula: C14H26N2O8

MW: 350.17 g/mol

 $m/z \ 351.24 \rightarrow m/z \ 333.33$  $m/z \ 351.24 \rightarrow m/z \ 140.10$  $m/z \ 351.24 \rightarrow m/z \ 98.00$ 



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Streptomycin

MW: 581.27 g/mol

 $m/z 582.17 \rightarrow m/z 263.09$  $m/z 582.17 \rightarrow m/z 246.05$  $m/z 582.17 \rightarrow m/z 176.00$ 

Formula: C21H39N7012



Tobramycin

Formula:  $C_{18}H_{37}N_5O_9$  MW: 467.26 g/mol m/z 468.36  $\rightarrow$  m/z 163.19 m/z 468.36  $\rightarrow$  m/z 145.10







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### K. APPROVALS AND AUTHORITIES

- 1. Approvals on file.
- 2. Issuing Authority: Director, Laboratory Quality Assurance Division.