

Determination of tobramycin using HPAE-IPAD on a compact ion chromatography system

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Keywords

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aminoglycoside, carbohydrate,
antibiotic, drug substance

Goal

To develop an HPAE-IPAD method for the determination of kanamycin A, kanamycin B, and tobramycin using a high-pressure-capable Thermo Scientific™ Dionex™ Integrion™ HPIC™ system.

Introduction

Tobramycin is an important aminoglycoside antibiotic used in ophthalmic and intravenous treatments to treat bacterial infections by blocking protein synthesis.¹ Tobramycin is isolated from the fermentation of the actinomycete *Streptomyces tenebrarius* with kanamycin A and kanamycin B present as impurities from either incomplete isolation of the drug or from degradation of tobramycin. Therefore, it is important to assay the tobramycin content and quantify the related impurities of a tobramycin-based antibiotic. Determination of tobramycin, kanamycin, and other aminoglycoside antibiotics has been previously demonstrated by High Performance Anion Exchange with Integrated Pulsed Amperometric Detection (HPAE-IPAD).²⁻⁶ These publications demonstrated the advantages of using eluent generation (EG) for this application.

This document updates Thermo Scientific™ Application Note 61 using a high-pressure-capable Dionex Integrion HPIC system equipped with updated EG technology and an electrochemical detector.

Experimental

Equipment

- Thermo Scientific Dionex Integrion HPIC System (P/N 22153-60305), including:
 - Temperature control in the detector and column oven chambers
 - EG capabilities
 - Eluent degas module
 - Device monitoring
 - Vacuum Degas Kit (P/N 00108-01-00046)
- Integrion ED detector (P/N 22153-62035) with Thermo Scientific™ Dionex™ Integrion electrochemical cell without electrodes (P/N 072044) (or the same P/N from the Thermo Scientific™ Dionex™ ICS-3000 IC, ICS-5000 IC or ICS-5000+ HPIC systems)
- Mobile Control Option (P/N 22153-62031) and Android tablet (P/N 22153-62100)
- Thermo Scientific™ Dionex™ AS-AP Autosampler temperature control option (P/N 074926) with 1.5 mL trays (P/N 074936) and 250 µL syringe (P/N 074306)

Reagents

- Degassed ASTM™ Type I deionized water,⁷ vacuum degassed with ultrasonic agitation
- pH buffer solutions to calibrate pH – Ag/AgCl Reference electrode: Fisher Scientific™ P/N SB115-500 (pH 7) and P/N SB107-500 (pH 10)
- Tobramycin (Sigma-Aldrich® Chemical Co, P/N T40014)
- Kanamycin B (Sigma-Aldrich Chemical Co, P/N B5264)

Software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2 SR4

Consumables

Table 1 lists the consumable products needed for a Dionex Integrion HPIC Reagent-Free IC capabilities, configured for electrochemical detection.

Chromatographic Conditions

Columns:	Thermo Scientific™ Dionex™ CarboPac™ PA1 guard (4 × 50 mm) and separation (4 × 250 mm)		
Eluent:	2 mM KOH		
Eluent Source:	Thermo Scientific™ Dionex™ EGC 500™ KOH cartridge with Thermo Scientific™ Dionex™ CR-ATC™ 600 trap column and high pressure EG degas module (P/N 075522)		
Flow Rate:	0.5 mL/min		
Column Temperature:	30 °C		
Detector Compartment Temperature:	30 °C		
Injection Volume:	20 µL, in PushFull mode		
Waveform:	<i>Time (s)</i>	<i>Potential (V)</i>	<i>Integration</i>
	0.00	+0.13	
	0.04	+0.13	
	0.05	+0.33	
	0.21	+0.33	Begin
	0.22	+0.55	
	0.46	+0.55	
	0.47	+0.33	
	0.56	+0.33	End
	0.57	-1.67	
	0.58	-1.67	
	0.59	+0.93	
	0.60	+0.13	
Detection:	IPAD, AAA-Direct Waveform vs pH, 1.67 Hz		
Working Electrode:	AAA-Direct disposable gold working electrode, 0.002" thick Teflon® gasket		
Reference Electrode			
Mode:	pH/Ag/AgCl in pH mode		
Run Time:	16 min		
Typical Background:	50–90 nC		

Table 1. Consumables list.

Product	Description	Part Number
Thermo Scientific™ Dionex™ IC PEEK Viper™ fitting tubing assembly kits	Dionex IC PEEK Viper fitting assembly kit for the Dionex Integrion HPIC system: Includes one each of P/Ns: 088805–088807, 088809, 088811	088797
Dionex IC PEEK Viper fitting tubing assemblies	Guard to separator column: 0.007 × 4.0 in (102 mm)	088805
	Injection Valve, Port C (Port 2) to guard column: 0.007 × 5.5 in long (140 mm)	088806
	EGC Eluent Out to CR-TC Eluent In: 0.007 × 6.5 in (165 mm)	088807
	Separator to ED Cell In: 0.007 × 7.0 in (178 mm)	088809
	CR-TC Eluent Out to Degasser Eluent In: 0.007 × 9.5 in (241 mm)	088811
Dionex AS-AP Autosampler vials	Package of 100, polystyrene vials, caps, blue septa, 10 mL	074228
Dionex AS-AP Autosampler vials	Package of 100, polypropylene vials, caps, 1.5 mL	079812
Thermo Scientific™ Dionex™ EGC™ 500 KOH Eluent Generator cartridge	Eluent generator cartridge	075778
EG Vacuum Degas Conversion Kit	Recommended for carbohydrate analysis methods using eluent generation. Included when ordering a Dionex Integrion HPIC system with ED detector	00108-01-00046
HP EG Degas module	Recommended for use with eluent generation. Included when ordering an Integrion with eluent generation	075522
Thermo Scientific™ Dionex™ CR-ATC™ 600 Electrolytic trap column	Continuously regenerated trap column used on Integrion systems with the Dionex EGC KOH 500 cartridge	088662
Thermo Scientific™ Dionex™ CarboPac™ PA1 Guard	Guard column, 4 × 50 mm	043096
Dionex CarboPac PA1 separation column	Separation column, 4 × 250 mm	035391
Thermo Scientific™ Dionex™ AAA-Direct disposable working electrodes	AAA-Direct carbohydrate gold working electrodes, preferred for this application	060082
Thermo Scientific™ Dionex™ Carbohydrate disposable working electrodes	Carbohydrate gold working electrodes, suggested alternative working electrodes for this application	060139
Thermo Scientific™ Dionex™ Ag/AgCl reference electrode	Reference electrode for this application	061879
Extra gaskets for working electrodes	Extra gaskets, PTFE, 0.002" thick	060141
Thermo Scientific™ Nalgene™ Rapid Flow™ sterile disposable filter units	1000 mL vacuum filtration flask, PES membrane, 0.2 µm,	5670020 (Fisher Scientific P/N 09-741-03)

Standard and eluent preparation

Use ASTM Type I deionized (DI) water for standards, eluent, and autosampler flush solution. It is important to degas the DI water to supply the eluent generator and to maintain 3–5 psi of inert gas headspace over the DI water eluent when running an HPAE-PAD method. Absorbed carbon dioxide gas can result in poor chromatography and variable retention times.

To prepare 2 L of the degassed ASTM Type I DI water to supply the eluent generator, degas 1 L of DI water by vacuum filtration (Nalgene, 1 L, PES, 0.2 μm) with applied ultrasonic agitation. Transfer the degassed water to the 2 L eluent bottle and cap the bottle. Prepare another 1 L in the same manner. Connect the 2 L eluent bottle containing degassed DI water to the Dionex Integrion HPIC system pump eluent line. Connect nitrogen or other inert gas to the eluent bottle to provide ~3–5 psi headspace pressure.

Weigh the tobramycin reagent and dissolve in DI water to prepare a 10 mg/mL stock standard. Use polypropylene volumetric flasks, storage containers, and autosampler vials to avoid sample losses. Tobramycin, and to a lesser extent kanamycin B, when dissolved in water adsorbs

to glass surfaces. Significant losses due to adsorption occur at dilute concentrations.² The tobramycin working standards were prepared by diluting the stock standard with DI water to 0.5, 1.0, 10, 50, and 100 μM . Kanamycin A and kanamycin B solutions (0.1, 1, 5, and 10 μM) were prepared in the same manner. For recovery experiments, 10 μM and 26 μM tobramycin solutions were prepared from the stock standards. Method detection limit (MDL) standards were prepared by serial dilution from the 0.1 μM working standard. The samples were stored at -20 °C and thawed prior to analysis.

Instrument setup and installation

The Dionex Integrion HPIC System with RFIC capabilities is a high-pressure-capable, integrated system. The Dionex Integrion HPIC system, the Dionex EGC 500 KOH cartridge and Dionex CR-ATC 600 consumable products are designed for high pressure conditions up to 5000 psi.

To set up this application, connect the Dionex AS-AP autosampler and the Dionex Integrion HPIC system, equipped with an electrochemical detector, according to Figure 1. Note that the injection valve is plumbed through different ports than previous Dionex IC systems. See Thermo Scientific TN 176.⁸

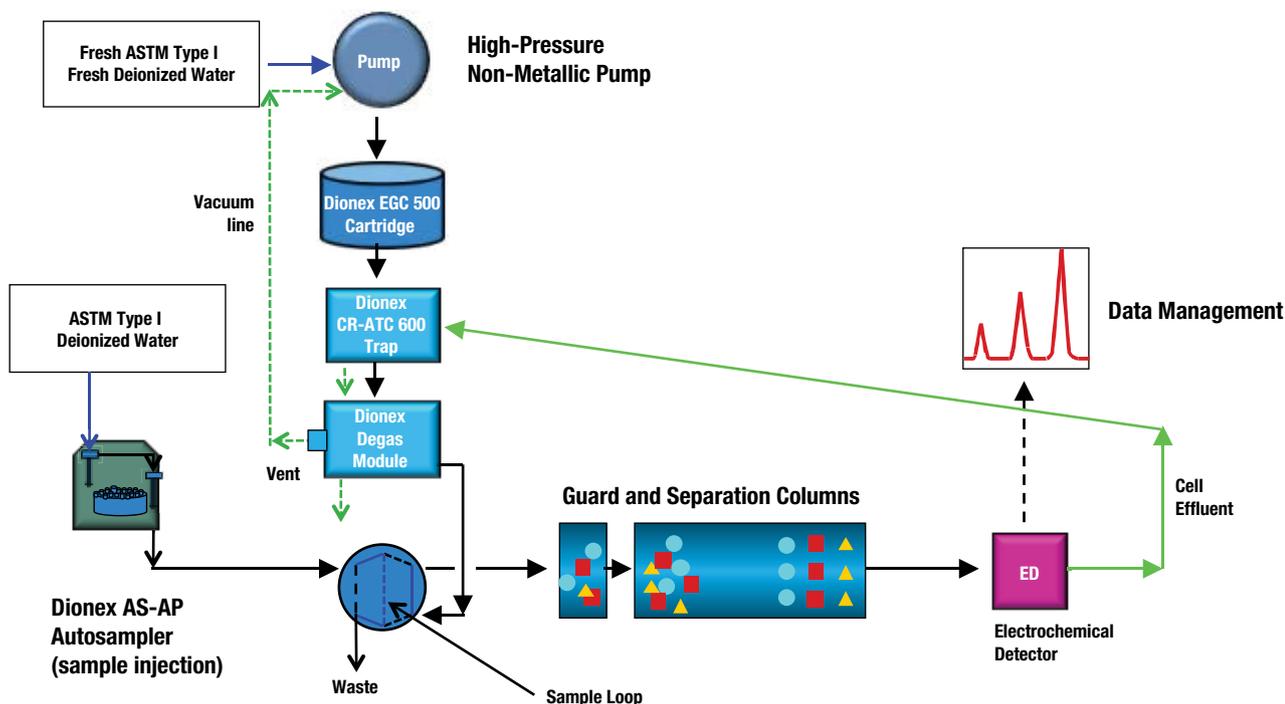


Figure 1. Flow diagram for the Dionex Integrion HPIC System configured for ED detection.

Connect the USB cables from the Dionex Integrion HPIC system to the Dionex AS-AP autosampler and to the computer. Connect the power cables and power on the IC instrument and the autosampler.

Configuring the modules in the Chromeleon CDS software

To configure the IC system:

1. Start the Chromeleon Instrument Controller program and select the link, *Configure Instruments* (opens Chromeleon Instrument Configuration Manager).
2. Right-click on computer name.
3. Select *Add an Instrument*, and enter an appropriate name (for example: Integrion_Tobramycin_1).
4. Select *Add a Module, IC: Dionex Integrated Modules, and Integrion HPIC System*.

In this application, only three modules are needed: the Dionex Integrion IC system, the autosampler, and the Integrion Pump Wellness module. The instructions to configure each module are summarized in Table 2.

To add the Dionex Integrion IC system in the configuration:

1. Right-click and select *Add a Module, IC: Dionex Integrated Modules, Integrion HPIC System* module.
2. Select USB address to link the module to the configuration.

The Chromeleon CDS software will automatically detect all Dionex Integrion IC system devices—the electrolytic devices, detectors, pump degasser, and seal wash—requiring minimal data entry during instrument configuration. The Chromeleon CDS software automates the system configuration process by automatically detecting these installed devices (Figure 2).

Table 2. Summary of system configuration for the Dionex Integrion HPIC system.

Tab	Action	Result
Dionex Integrion HPIC Module		
General	Link to USB address	
Pump		Flow Rate and pressure limitations are displayed
Detectors		Automatically detected
Electrolytics		Automatically detects Dionex eluent generator cartridges, and Dionex CR-TC trap columns (Figure 2)
Inject Device		Automatically detected
Thermal Controls		Automatically detects thermal control options for column, and detector
High-Pressure Valves		Automatically detected
Low-Pressure Valves		Not needed for this application, but automatically detected.
Options		Automatically detects Pump Degasser and Seal Wash pump
Pump Wellness Module		
Devices	Click pressure signal box	Activates pressure monitoring feature (Figure 3)
Dionex AS-AP Autosampler Module		
Add module	Link to USB address	
Sharing		Only if more than one instrument is detected. If this option is present, select Instrument
Segments / Pump Link		Select 10 mL polystyrene vials or 1.5 mL vials for “Red”, “Blue”, and “Green”
Options		Select Push, select syringe size, select 1.2 mL buffer line, enter the loop size

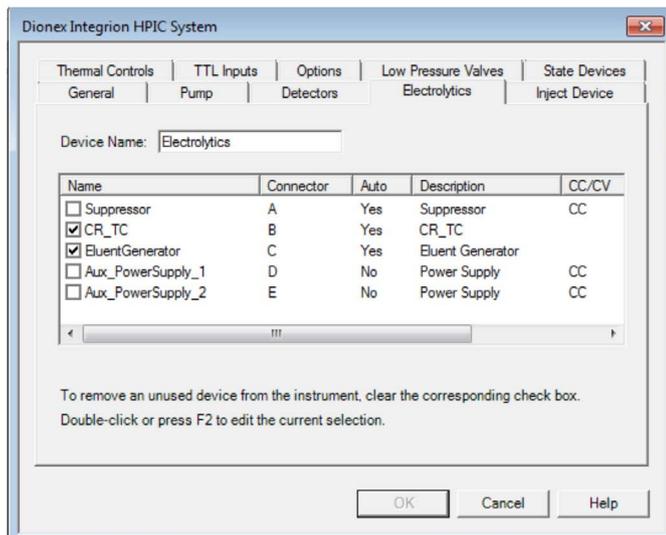


Figure 2. Automatic detection of electrolytic devices in the Dionex Integrion HPIC System module.

Adding the Dionex AS-AP Autosampler to the configuration

1. Add the Dionex AS-AP Autosampler as a module, and select the USB address.
2. In the Segments/Pump Link tab, select the appropriate vial trays for each color zone.
3. In the Options tab, select Push, installed syringe size, 1.2 mL for buffer line, and enter the sample loop volume (20 μ L).
4. Save the configuration.
5. Select *Check the Configuration*.
6. Close the Chromeleon Instrument Configuration program.

Plumbing the Dionex Integrion HPIC System

Decontaminate the IC system prior to installing the columns by pumping a 500 mM NaOH solution at 0.5 mL/min from the pump to valves for 2–3 h, or overnight if the system was previously used for another application. Finish the decontamination process by pumping DI water at 0.5 mL/min for 30 min.

To achieve the best chromatography, use the Dionex IC PEEK Viper fitting assemblies (listed below) and tighten to finger-tight. It is important that they are not overtightened. These fittings are used in the following locations:

- Dionex EGC 500 KOH eluent generator cartridge - *Eluent Out to Eluent In* on Dionex CR-ATC 600 trap column
- Dionex CR-ATC 600 trap column - *Eluent Out to Eluent In* on the Dionex Degas Module
- Injection Valve - Port 2 (Column) to the guard column
- Between the guard and separation columns
- Separation column to *Eluent In* on the ED electrochemical cell

To add pressure monitoring capabilities:

1. Right-click and select *Add a Module, IC: Dionex Integrated Modules, Integrion HPIC Pump Wellness* module.
2. Select USB address to link the module to the configuration.
3. Select the Devices tab and click on the pressure signal box (Figure 3).

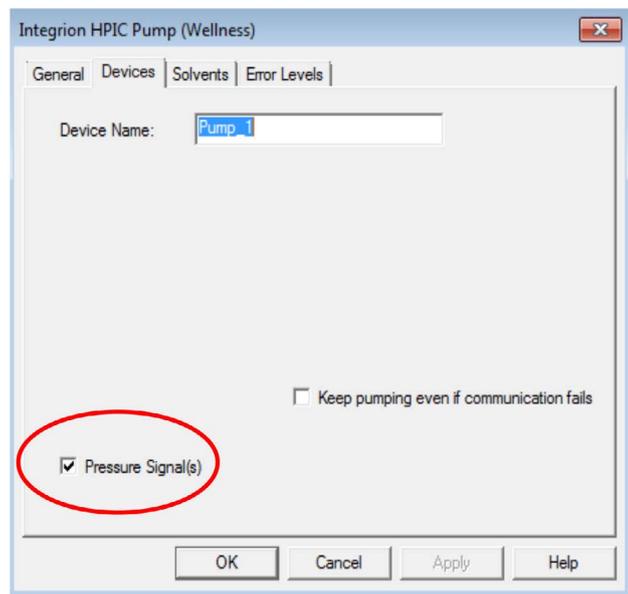


Figure 3. Adding the Dionex Integrion HPIC Pump Wellness module to instrument configuration.

To install an HPAE-IPAD application on the Dionex Integrion HPIC system, follow the instructions in Technical Note 176.⁸

1. Loosen the waste lines, including the metal-wrapped waste line in the back of the instrument, and direct the free ends to a waste container.
2. To plumb the system, first connect the pump eluent line to the eluent bottle containing DI water previously degassed (vacuum filtration and ultrasonic agitation).
3. Apply 3 to 5 psi of nitrogen or other inert gas headspace to prevent carbon dioxide absorption.
4. Prime the pump by opening the priming knob ¼ turn and pressing the priming button.
5. Prime the pump until no bubbles are visible and water is flowing at a steady rate out of the pump waste line.
6. Turn off the pump priming and close the priming knob to finger-tight.

For more information, review the Dionex Integrion HPIC system Installation and Operator's manual.⁹

Install vacuum line to degasser module vent

Hydroxide eluents produced by eluent generation require inline vacuum degassing to achieve the optimum conditions for electrochemical detection. Vacuum degassing is accomplished by connecting the vent line of the degas module to the vacuum pump port on the back of the instrument (Figure 4). In the Dionex Integrion HPIC system configured with an electrochemical detector (ED), the vacuum pump connector is pre-installed, eliminating the manual installation previously required.

At the start of the application, to ensure that the degasser pump is working properly, reset the pump degasser to "Off" and then "On".

1. Press the "F8" key while on the instrument console page, which brings up the manual commands.
2. Place the cursor on the pump and set Degasser to "Off".
3. Close the dialogue window and repeat with "On".

The degasser pump should turn on for a short time and then shut off, indicating that the degasser pump is working and that the vacuum is acceptable.

To assist in degassing the eluent, install a ¼" i.d. air tubing from the EG Degas module (P/N 075522) vent port to the vacuum connection in the back of the Dionex Integrion HPIC system (Figure 4). The degasser pump will turn on again for a short time to re-establish the vacuum. If the degasser pump stays on for longer than 1 min, tighten the fitting (Figure 4) ¼ turn.

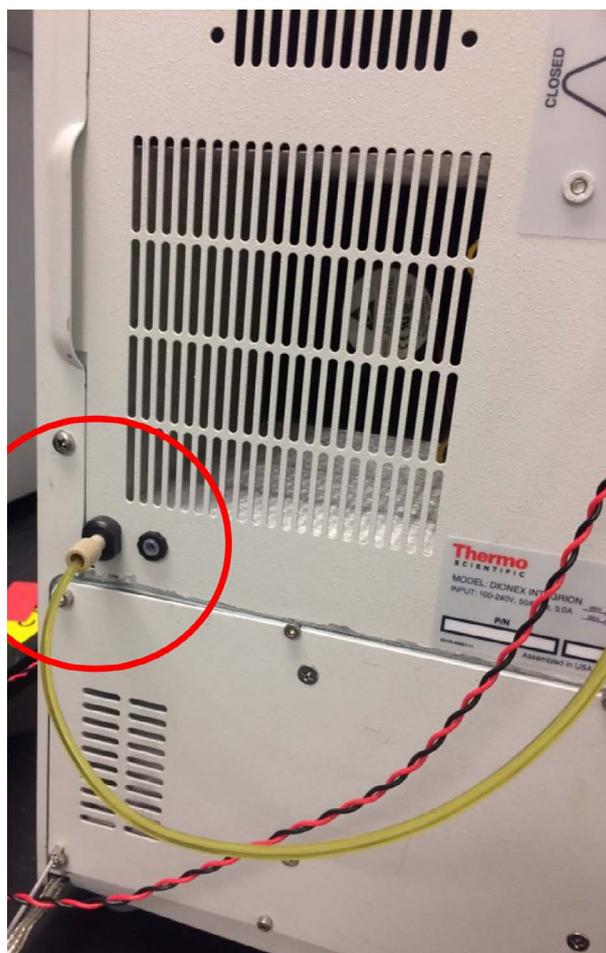


Figure 4. Installing the vacuum connection on the Dionex Integrion HPIC system.

Conditioning electrolytic devices and columns

Do not remove the tags on the columns and consumable devices. These tags are required for consumable device monitoring functionality.

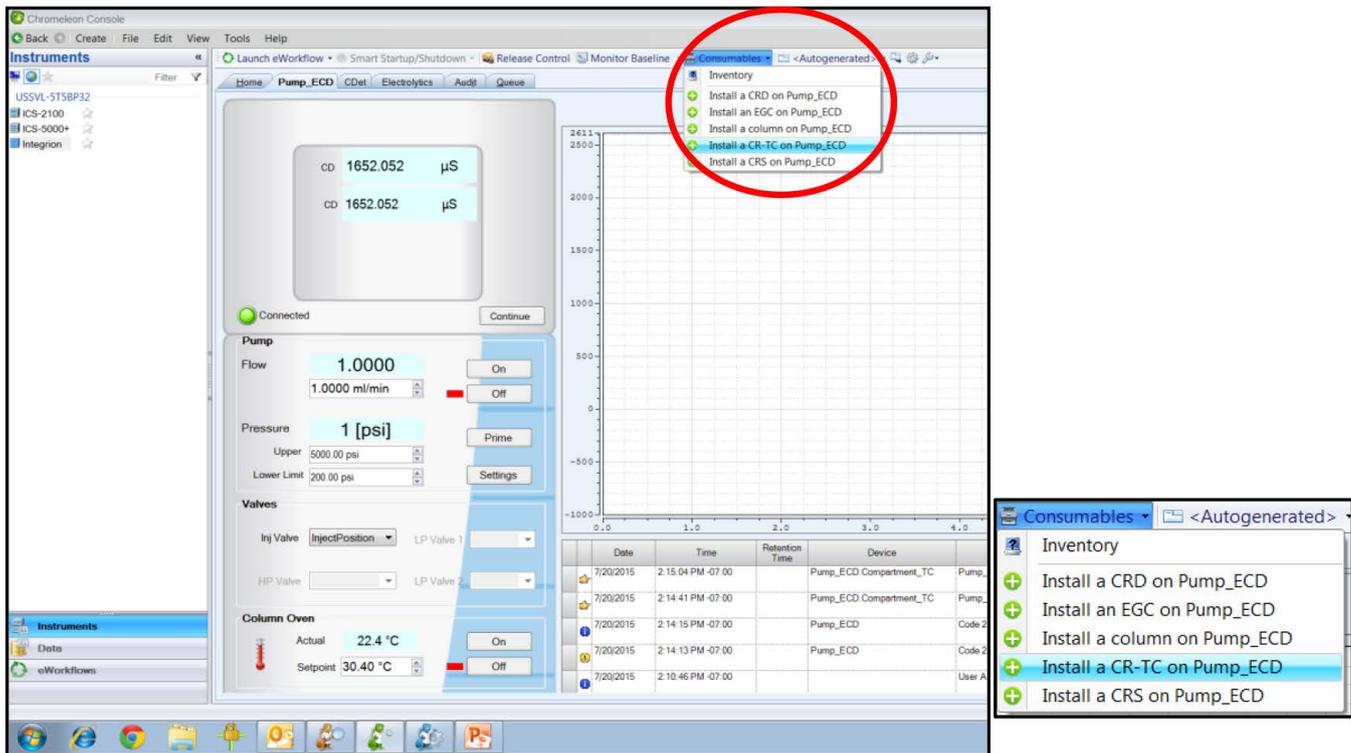


Figure 5. Consumables online installation instructions.

Install the Dionex EGC 500 KOH cartridge and Dionex CR-ATC 600 Continuously Regenerated Anion Trap Column in the Reservoir Tray compartment. Condition the devices according to instructions in the drop-down menu under Consumables, Install (Figure 5). (This information is also available in the product manuals and the system installation manual.⁹⁻¹³)

Condition the columns for 30 min according to the instructions from the Consumables, Install Column section (Figure 5). The general practice is follow the eluent and flow rate conditions listed in the column's Quality Assurance Report (QAR) while directing the eluent exiting the column to a waste container. Complete the installation according to the flow diagram in Figure 1.

Installing and optimizing the Dionex AS-AP Autosampler

The Dionex AS-AP Autosampler needle must be aligned to the injection port. To align the autosampler needle:

1. Select the Sampler tab on the instrument panel, and press the Alignment button.
2. Follow the commands to align the autosampler needle to the Injection Port and Wash Port (Section B.12 in the Operator's Manual).¹²

3. Connect the autosampler syringe line to wash container containing degassed DI water to the syringe.
4. Prime the syringe to flush out any air in the Buffer Wash line and syringe.
5. Initially select a 5000 μL wash volume until a steady flow of water is observed at the Wash Port. Reset wash volume to 100 μL .
6. Calibrate the transfer line volume by following the prompts on the TLV Calibration button. This volume will be recorded automatically.

For more information review Section 5.9 in the Dionex IC Series AS-AP Autosampler Operator's Manual.¹²

Electrochemical cell

Always wear particle-free nitrile gloves (such as Fisher Scientific P/N 19-130-1597 series) when handling the electrochemical cell. If this is a new ED Cell, disassemble the cell and discard the shipping gasket. **Caution:** Do not touch the working electrode with any paper products, as this can contaminate the working electrode. Avoid wrinkles in the gasket.

The ED Cell is a three-electrode cell: the cell body as the counter electrode, a reference electrode (pH-Ag/AgCl), and a working electrode (conventional or disposable). The fully assembled cell also includes a Yoke Block Assembly (with a support block) to tighten the cell and gaskets for the working electrode. The installation procedures mentioned below describe an electrochemical cell with an AAA-Direct gold disposable working electrode.

Installing the disposable electrode

1. Rinse the cell body, the walls of the reference electrode, and the inlet tube thoroughly with DI water.
2. Shake off the excess water, and dry with a lab wipe.
3. Select the working electrode for the application and corresponding gasket and support block. Rinse them with DI water and shake off the excess water.
4. Dry the gasket (0.002" thick Teflon) and support block with an absorbent tissue.
5. Assemble the cell according to the Operator's manual⁷ and ED User's Compendium for Electrochemical Detection¹³ by first installing the working electrode gasket flat against cell body. Avoid any wrinkles in the gasket, as this will cause a poor fit and subsequent leaks and poor detection. To minimize the chance of using a worn gasket and developing a leak, the gasket should be replaced after three compressions (i.e. disposable electrode installations).
6. Install the AAA-Direct disposable working electrode with the metal face down over the gasket.
7. Install the support block firmly over the working electrode.
8. Install the Yoke Block by squeezing the tabs and sliding it on the cell body.
9. Align the Yoke Block parallel to the cell body and rotate the Yoke Block knob clockwise until you hear three "clicks". (The cell with a conventional working electrode is assembled similarly with appropriate gasket, except the support block is not used.)

Calibrating and installing the pH-Ag/AgCl reference electrode in the electrochemical cell

To calibrate the reference electrode:

1. Condition the pH-Ag/AgCl reference electrode by removing the storage cap, rinsing the electrode with DI water to remove the potassium chloride storage solution, and then placing the electrode in a solution of pH 7 buffer.
2. Select pH buffer 7 and the corresponding buffer for the application, pH 10 for basic eluents and pH 4 for acidic eluents.
3. Install the cell into ED module and connect the yellow cable to the yellow port.
4. Install the reference electrode blue cable into the black port.
5. Immerse the reference electrode in pH 7 buffer to at least mid-level of the electrode.
6. Select the "pH Calibration" button on the ED Panel and follow the instructions to calibrate the electrode including using pH 10 buffer.
If calibration fails, it will be reported in the audit trail.

To install the reference electrode into the cell:

1. After calibration is completed and to make the installation more convenient, first remove the reference electrode and cell body from the ED module.
2. Rinse the buffer solution off the electrode with DI water, and gently, but firmly, screw-in or rotate the reference electrode clockwise into the reference electrode port of the electrochemical cell until the reference electrode is finger tight.
3. Immediately complete the final plumbing as described below.

Completing the plumbing

For optimum cell performance, use 2–3 ft (60–90 cm) of black PEEK tubing or 1–2 in (5–10 cm) of red PEEK tubing at 0.5 mL/min for optimum cell backpressure of ~12 psi at 0.5 mL/min.

Caution: Excess tubing can cause band-broadening and thus reduce detection response.

The cell waste line should be removed prior to each reference electrode installation and installed after flow has started through the cell. (See discussion below.)

Complete the installation:

1. Install the cell in the ED detector module and connect the reference electrode cable (blue to black) and the counter/working electrode cable (yellow to yellow).
2. Remove the temporary waste line from the column and install the IC PEEK Viper fitting (P/N 088809, 0.007 × 7.0 in) to the column outlet.
3. Allow liquid to flow from the end of the column.
4. Connect the free end to the cell inlet tube.
5. Allow the liquid to flow from cell exit hole and then connect a 2 to 3 ft piece of black PEEK tubing (0.010 in, 0.254 mm i.d.).
6. Wait 60 s before tightening the connection. The tubing provides ~12 psi backpressure (at 0.5 mL/min) on the cell to prevent outgassing and detector noise. (The comparable length of red PEEK (0.005 in, 0.013 mm i.d.) tubing is 1–2 in.)
7. Connect the cell waste tubing to the CR-ATC Regen In line.

Starting the Dionex Integrion HPIC System

To start the system:

1. Turn on the pump.
2. Immediately turn on both the Dionex EGC 500 eluent generator cartridge and the Dionex CR-ATC 600 trap column when liquid is flowing through the device.

The system backpressure is dependent on the flow rate and type of column but must be above 2000 psi to support the EGC cartridges.

3. Install yellow PEEK backpressure tubing (yellow PEEK, 0.076 mm i.d., 0.003 in i.d.) between the HP EG Degas module and the injection port (Port 1, Pump position).

This application may require up to 1500 psi delivered by backpressure tubing.

4. Set the eluent concentration, column oven, compartment oven, and cell temperatures as shown in the Conditions section of the application.
5. Turn on the ED cell after the pH > 10.
6. Select Integrated Pulsed Amperometric for cell mode.
7. Select AAA-Direct waveform.
8. Enter the data collection frequency and the “pH” as reference electrode mode into the ED Panel.
9. Allow the system to equilibrate for 30 min.

For optimum chromatography equilibrate until the total background is stable, ~5 nC/min.

Creating an instrument sequence and instrument method

Use the Thermo Scientific™ AppsLab™ Library of Analytical Application database to download the Chromeleon sequence.¹⁴

To download the sequence:

1. Open AppsLab database.
2. Search for the title of this application note or search by using the key words, tobramycin and Integrion.
3. Select the AppsLab record.
4. Select the Downloads tab.
5. Select the eWorkflow to automatically generate the Chromeleon sequence.

Use Chromeleon Wizard to create a new method and a new sequence.

To create a new instrument method:

1. Select *Create, Instrument Method*, and select *Instrument* (such as *Integrion_Tobramycin_1*).
2. Enter the values from the Chromatographic Conditions section.
3. Save the instrument method.

To create a new sequence:

1. Select *Create, Sequence*, select the instrument, select ED or ED_Total as the preferred channel.
2. Save the sequence.

Device monitoring

A new feature of the Dionex Integrion HPIC system is the device monitoring and tracking, which automatically detects the electrolytic devices and the columns.

Review and approval of the devices is required to start the first sequence on the Dionex Integrion HPIC system and after installing new consumable devices.

To access this approval:

1. Select Consumables and select Inventory (Figure 6).
The device monitoring shows the device history, tracking: Part No., size, serial numbers, manufacture lot, installed location (On Device), and Best if Use by Date (Figure 6, top). Additionally, the device monitoring will provide warnings if there is incompatibility in the devices installed (Figure 6, bottom left).
2. To start the sequence, review the list of consumables listed as inventory.
3. Correct any errors and approve.
4. Close the page (Figure 6, bottom right).
5. Select the Instrument Queue tab.
6. Conduct a Ready Check on the sequence.
7. Press Start.

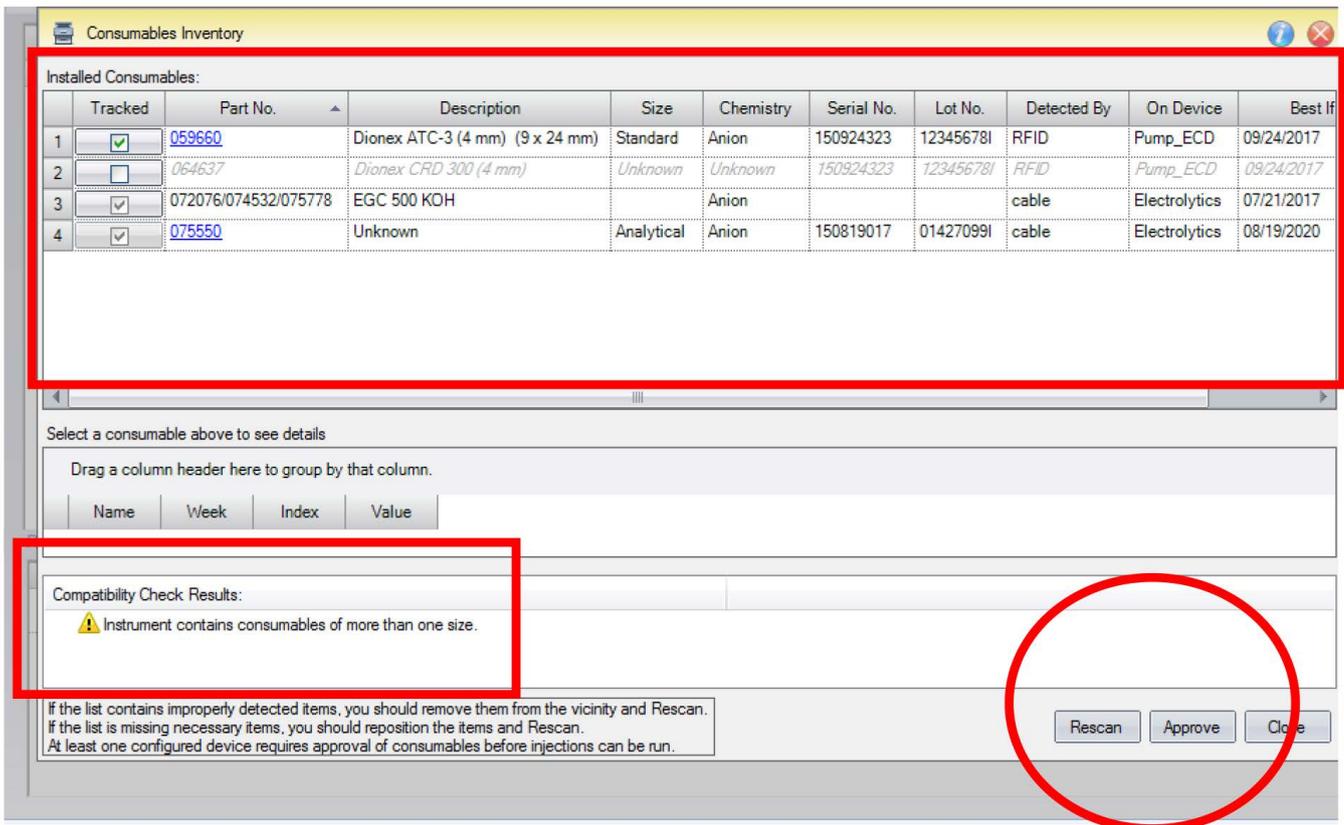


Figure 6. Consumables tracking.

Results and discussion

Tobramycin and other aminoglycoside determinations were previously demonstrated in AN61 using HPAE-PAD or IPAD with an IC system, autosampler, and EG device. Since AN61 was published, there have been many technology advances in IC instruments, consumables, and detectors. This application update demonstrates the same application executed on a Dionex Integrion HPLC system with an ED detector and ED cell, the latest technology in eluent generation consumables, and a Dionex AS-AP Autosampler.

Tobramycin and the aminoglycoside impurities were separated by an electrolytically generated eluent (2 mM KOH). Electrolytic eluent generation provides accurate eluent concentrations and precise retention times. The aminoglycosides were separated at 0.5 mL/min on the Dionex CarboPac PA1 anion-exchange column and detected by IPAD using the AAA-Direct waveform.

Figure 7 shows the chromatogram of tobramycin with kanamycin impurities. The run-time was extended two minutes from the AN61 conditions to 16 min to ensure that the oxygen dip (a dip in the baseline that is result of having less dissolved oxygen in the sample than in the eluent) did not appear near a peak of interest. The oxygen dip (~31-min retention time) is due to oxygen present in the samples and appears as a function of the gas permeation volume of the column. Like some organic impurities, eluting oxygen produces less background than the eluent, so there is a dip in the baseline. The retention times of the oxygen dip and other baseline dips vary from column to column, and depend on the flow rate, not the eluent strength. Eluting the baseline dips just prior to the end of run, or timing their elution to occur at the end of the following injection, prevents the baseline dips from interfering with the peaks of interest. The tobramycin peak exhibits some asymmetry and a small baseline slope after it elutes, but does not interfere with integration. At the low eluent conditions, carbonate can accumulate on the column resulting in a reduction of analyte retention time. A prudent practice is to run a column wash in the morning at 2 mM KOH for 16 min and

Column: Dionex CarboPac PA1 guard, 4 × 50 mm
Dionex CarboPac PA1, 4 × 250 mm
Eluent: 2 mM KOH
Eluent Source: Dionex EGC-500 KOH cartridge,
with Dionex CR-ATC 600 trap column,
Dionex high pressure degasser
Flow Rate: 0.5 mL/min
Column Temperature: 30 °C
Detector Comp.: 30 °C
Injection Volume: 20 µL
Detection: IPAD, AAA-Direct Au disposable electrode,
0.002" thick gasket
Reference Electrode: pH/Ag/AgCl, pH mode
Waveform: AAA-Direct, versus pH, 1.67 Hz
Peaks:
1. Void volume
2. System peak
3. Kanamycin A
4. Kanamycin B
5. Tobramycin
6. Oxygen dip

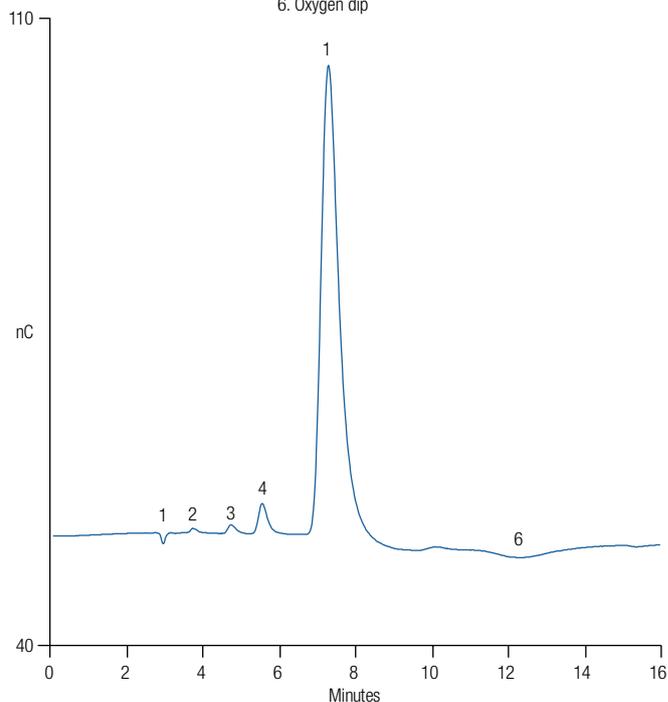


Figure 7. 20 µM tobramycin with trace amounts of kanamycin A and B.

65 mM KOH for another 44 min. It may be necessary to add another column wash during the day, but it was not found to be necessary during these experiments.

The peak response of the tobramycin was also evaluated using the Four Potential Carbohydrate waveform. The response is significantly lower than with the AAA-Direct waveform, as previously reported in AN61, but if desired, the carbohydrate waveform can be used as demonstrated and discussed in AN61.

Method qualification

The tobramycin method was evaluated on the Dionex Integriion HPIC system using the previously described conditions. Additionally, general system suitability practices were followed as stated in USP® General Chapter <621> Chromatography.¹⁵ The method was evaluated for reproducibility, linearity, accuracy, and sensitivity. The short-term stability was evaluated by determining the reproducibility of triplicate injections of 20 µM tobramycin and two of the impurities, 10 µM kanamycin A and 5 µM kanamycin B. The experiments showed good reproducibility with RSDs of 1.68, 0.46, 0.14%, respectively, for tobramycin, kanamycin A, and kanamycin B.

The peak response to concentration (linearity) was evaluated from 1–100 µM (0.5, 1.0, 10, 50, and 100 µM) for tobramycin and 0.1–10 µM (0.1, 1, 5, and 10 µM) for kanamycin A and kanamycin B. The responses were linear for both kanamycin analytes with coefficients of determination, $r^2 > 0.999$). These results were similar to those reported in AN61. In contrast to previous reports, the best correlation was obtained for tobramycin by applying a quadratic fit (Figures 8A, 8B, 8C).

Sensitivity was estimated by calculating the limit of detection (LOD) using nine replicate injections of a 0.010 µM tobramycin standard (S/N 24), diluted serially from 1 µM tobramycin working standard. The estimated LOD at $3 \times S/N$ was 1.3 nM (0.026 pmol on column, 20 µL injection), which is comparable to previously reported values.

The LODs for kanamycin A and kanamycin B were determined similarly using 10-fold dilution of 0.1 µM working standards. The results were 14 (0.29 pmol on column) and 15 µM (0.30 pmol on column), respectively.

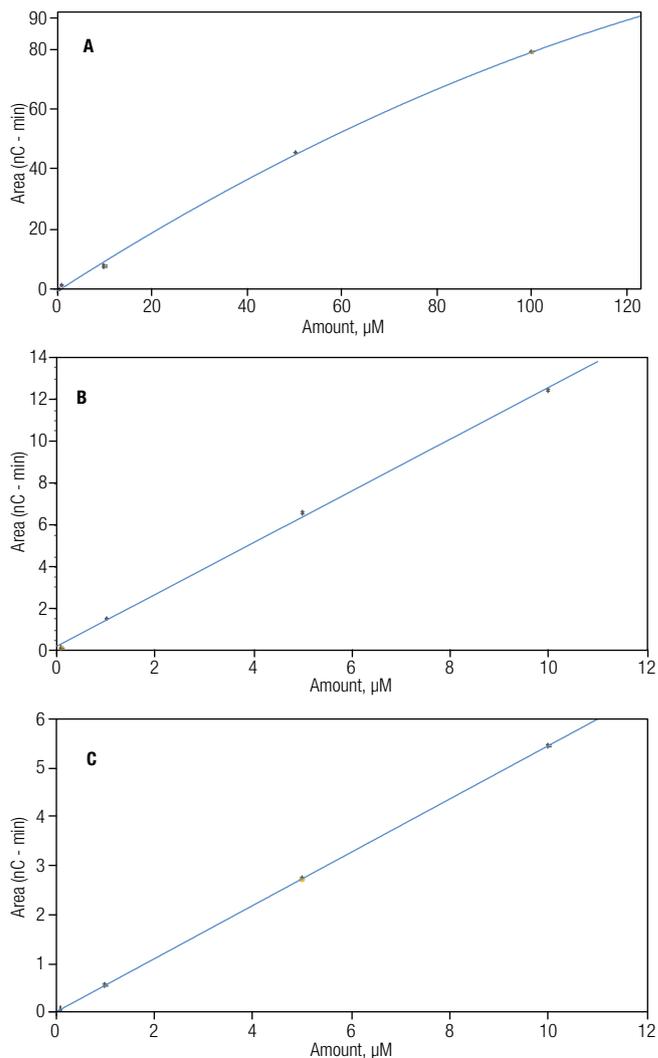


Figure 8. Calibration curves for (A) tobramycin, (B) kanamycin A, and (C) kanamycin B.

The method accuracy was determined by the average percent recovery of six replicate injections of 16.8 ± 0.24 µM tobramycin added to a 10.05 ± 0.10 µM standard. The experiments averaged 98.6% recovery, indicating that the method was accurate.

Conclusion

Determination of kanamycin A, kanamycin B, and tobramycin were demonstrated on a Dionex CarboPac PA1 anion exchange column by HPAE-IPAD. In this update, previously published AN61 is demonstrated on the Dionex Integriion HPIC system. Additionally, detailed instructions were added to support successful installations and operation.

The method was evaluated for linearity, reproducibility, and sensitivity which were found to be comparable to the values previously reported in AN61. All of the aminoglycoside antibiotic compounds evaluated had higher responses using the AAA-Direct waveform (Gold, AAA) than the Four Potential Carbohydrate waveform. However, as reported in AN61, the Four Potential Carbohydrate waveform (Gold, Carbo, Quad) is also suitable when pmol sensitivity is not needed.

More information on this application, including downloadable instrument methods, can be accessed through the Thermo Scientific AppsLab Library of Analytical Applications.¹⁴

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