Agilent

Agilent Buffer Advisor

User's Guide

Notices

Document Information

The information in this document also applies to 1260 Infinity II and 1290 Infinity II modules.

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Contents

1 Introduction to Buffer Advisor

This chapter provides an overview of the Agilent Buffer Advisor software.

What is Buffer Advisor?

Buffer Advisor is a specialized utility software designed to simplify ion-exchange chromatography workflows and support design of experiments (DoE). It integrates seamlessly with OpenLab, providing users with a streamlined approach to creating pH and salt gradients.

The software eliminates the labor-intensive and error-prone steps traditionally associated with buffer preparation, buffer blending, and pH scouting. With Buffer Advisor, users can quickly set up salt or pH gradients using up to four stock solutions, optimizing ionic strength or pH for various chromatography techniques such as size exclusion (SEC), ion exchange chromatography (IEX), hydrophobic interaction (HIC), and affinity chromatography.

The process involves defining the buffer concentration, pH, and maximum salt concentration, selecting suitable stock solution concentrations, and verifying the buffer system's compatibility with the separation task. Finally, Buffer Advisor automatically generates a pump timetable for the desired gradient, significantly reducing preparation time and enhancing method development efficiency.

The mixing principle of Agilent Buffer Advisor is depicted in Figure 1 on page 5 for salt gradients, and Figure 2 on page 6 for pH gradients.

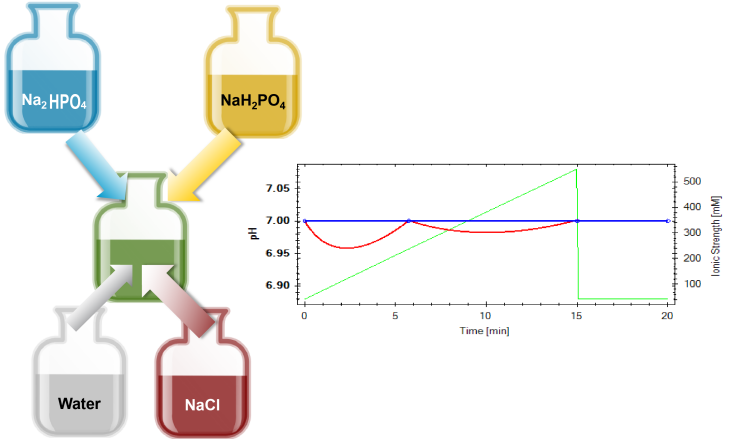


Figure 1: Quaternary mixing of four stock solutions (water, salt solution, acid and base) with the Agilent Bio-inert LC and Buffer Advisor generates a linear salt gradient at constant pH.

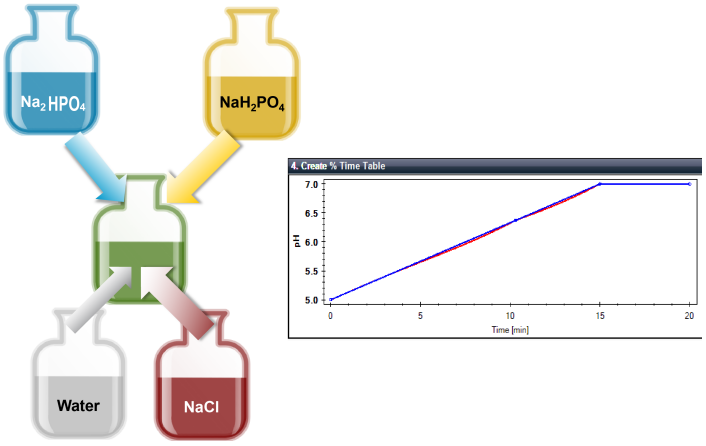


Figure 2: Proportioning of three solutions (water, acid, base) with Agilent Bio-inert quaternary LC and buffer Advisor results in formation of a linear pH gradient.

Quaternary solvent mixing using the Agilent Buffer Advisor provides the capability of automatically forming several mobile phases of different pH values for different methods from four stock solvents. This enables automated method development (pH scouting) by screening mobile phases for an analyte separation at different pH values without the necessity to prepare the different buffers manually. In addition, the Agilent Buffer Advisor software corrects a gradient timetable with compensation points (added timetable steps), ensuring that the pH is kept constant while the ionic strength is changing. The timetable can be imported into the Agilent LC&CE Instrument Drivers of quaternary Bio and Bio-inert pumps.

The result is displayed as a pump timetable in the user interface of the respective Chromatography Data System (CDS) method.

2 A Short Excursion into Ion Exchange Chromatography

This chapter provides a short introduction to the challenges posed by ion exchange chromatography and how you can use the Agilent Buffer Advisor software to solve them.

Properties of Proteins

Proteins consist of amino acids with both acidic and basic functional groups, leading to a net positive, neutral, or negative surface charge. Due to differences in the pKa values of the basic and acidic amino acids, the net charge of a protein depends on the pH of the surrounding medium. At high pH, the net charge of the protein becomes more negative, whereas at low pH, it becomes more positive. The pH at which the net charge of the protein is zero is called the *isoelectric point*, *pI*.

In ion exchange chromatography, two approaches can be used to exploit the net charge differences of proteins for separation:

- **Salt Gradients:**

  The most commonly used method, where increasing ionic strength leads to the separation of proteins. When a salt gradient is used, the ionic strength of the eluent is increased to elute the proteins in the order of increasing binding strength to the column.

- **pH Gradients:**

  An alternative method that avoids the necessity of applying highly concentrated salt solutions. If a pH gradient is used, the proteins elute at the pH where the net charge is zero, at their isoelectric points.

In ion exchange chromatography, knowledge of a protein's pI is crucial for selecting the starting conditions to ensure that the protein interacts with the stationary phase. A mobile phase pH below the pI of the protein of interest allows the use of a cation exchange column, whereas a mobile phase pH above the pI enables protein separation on an anion exchange column. In both cases, the protein binds to the column according to its current charge. A negatively charged column is used in cation exchange, whereas a positively charged column is used in anion exchange.

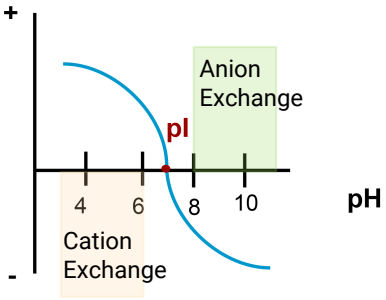


Figure 3: Isoelectric point (pI) of a theoretical protein of 7. If the protein is stable above its pI, Anion Exchange is recommended, if it is stable below its pI, Cation Exchange is recommended.

Table 1 on page 9 shows a list of proteins and their pIs. A buffer system and an appropriate column are selected for the analytical task depending on the pI of the protein. As a rule of thumb, proteins with high pI are separated on cation-exchange columns, proteins with low pI on anion exchanger columns.

Table 1: List of example proteins and their pIs

Protein	pI	Source
Amyloglucosidase	3.6	Aspergillus niger
Ovalbumin	4.7	Chicken, egg white
BSA	4.9	
Human insulin	5.4	
Bovine carbonic anhydrase	6.5, 5	Cow, erythrocyte
Myoglobin	7.4, 6.9	Horse, muscle
Ribonuclease A	8.88	Cow, pancreas
Cytochrome C	10.7, 9	Horse, heart
Lysozyme	11.3	Chicken, egg white

Ion Exchange: Salt Gradient

A salt gradient triggers a competitive process in cation exchange chromatography. In this technique, positively charged proteins bind to a negatively charged solid support. The mobile phase buffer is maintained at a constant pH below the protein's isoelectric point (pI) to ensure the protein's net charge remains positive. Salt ions (e.g., NaCl) serve as competing agents; at low salt concentrations (low ionic strength), the protein binds to the column. As the salt concentration increases, the positively charged sodium ions displace the positively charged proteins, resulting in their elution. Proteins with a higher net positive charge will elute later in the gradient.

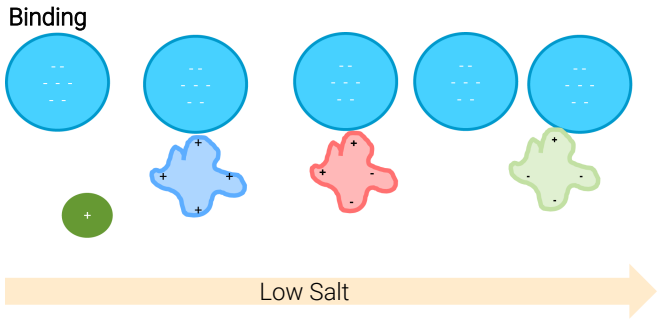


Figure 4: In a first step, positively charged proteins are bound to the column.

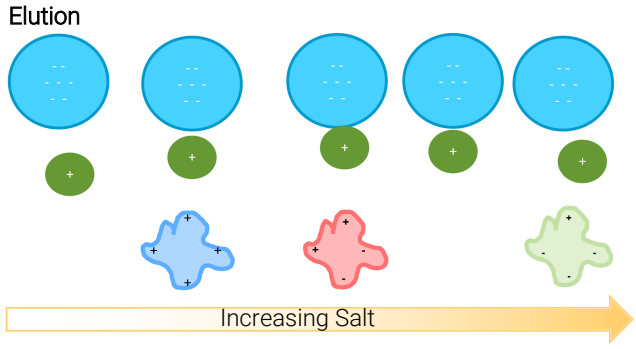


Figure 5: The salt concentration is increased and proteins elute.

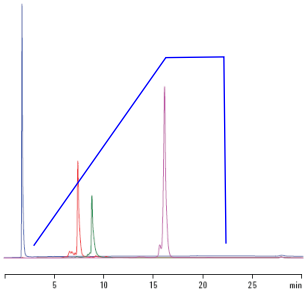


Figure 6: Example chromatogram of Cation Exchange chromatography employing a salt gradient. Ovalbumin, Ribonuclease A, Cytochrome C, Lysozyme were separated in pH 6.5 phosphate buffer. Protein separation with a salt gradient from 0–800 mM NaCl, 20 mM phosphate buffer, pH 6.5, automatically generated by the Agilent Buffer Advisor software.

Table 2: Conditions

Columns	Agilent Bio WCX NP10, 4.6 × 250 mm SS Agilent Bio WCX NP5, 4.6 × 250 mm SS
Mobile phase	A: Water B: 1.6 M NaCl C: 40.0 mM NaH_2PO_4 D: 40.0 mM Na_2HPO_4 By combining predetermined proportions of C and D, 20 mM buffer solutions at the desired pH range were produced.
Gradient	0 – 50 % B, 0 – 20 min 50 % B, 20 – 25 min 0 % B, 25 – 35 min
Flow rate	1.0 mL/min
Temperature	Ambient
Sample	Ovalbumin, Ribonuclease A, Cytochrome C, Lysozyme
Detection	UV, 220 nm
Instrument	Agilent 1260 Infinity Bio-inert HPLC system

Ion Exchange: pH Gradient (Chromatofocusing)

A pH gradient consists of a starting buffer and an elution buffer. In the starting buffer, the net surface charge of the protein should be such that it can interact with the functional group of the ion exchange resin. For example, in cation exchange chromatography, the starting pH of the buffer system is low. As the pH is increased linearly, the amino acids of the protein deprotonate, reducing their positive charge. When the pH of the mobile phase matches the isoelectric point (pI) of the protein, the protein no longer interacts strongly with the resin and elutes from the column. This results in the formation of a pH gradient across the column. Proteins with lower pI values (and thus less positive charges at the starting pH) will elute earlier than those with higher pI values (and more positive net charges) (see Figure 7 on page 14).

In contrast, in anion exchange chromatography, the starting conditions are at a high pH, and the pH decreases linearly during the chromatographic run. This causes the amino acids of the protein to protonate, reducing their negative charge. When the pH matches the pI of the protein, it elutes from the column.

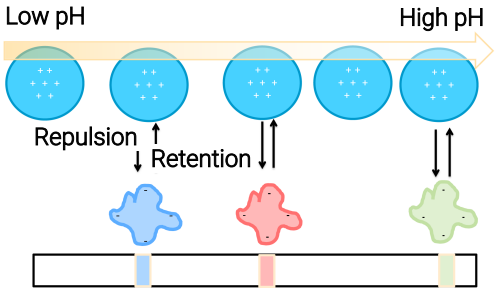


Figure 7: Example of a pH gradient: Proteins with same pI are focused in bands on the column.

The advantages of ion-exchange chromatography (IEX) with a pH gradient are threefold:

- Improved resolution: A pH gradient allows for more precise separation of proteins based on their isoelectric points (pI), leading to better resolution of protein peaks.
- Correlation of pI to buffer pH: The pI of the protein can be correlated to the pH of the buffer system, facilitating more accurate identification and characterization of proteins.

- Avoidance of salt solutions: By using a pH gradient, salt solutions can be avoided, reducing ionic interactions and preventing corrosion of the chromatography equipment.

While a pH gradient is preferable, forming a linear pH gradient over a wide pH range is challenging because a single buffer system typically provides sufficient buffer capacity only over a narrow pH range.

The Buffer Advisor software is an ideal tool that helps to select a suitable buffer system for a specific separation task.

3 Columns and Buffers

This chapter gives some background information on the selection of columns and buffers for ion exchange chromatography.

Column Selection

The column and separation buffer are selected depending on the charge of the protein. In ion exchange chromatography, the pH of the mobile phase buffer must be between the pI or pKa of the charged molecule and the pKa of the charged groups on the stationary phase.

In *anion exchange chromatography*, a strong or a weak anion exchange column containing a quaternary ammonium ion, or a weak anion exchanger with either a tertiary or secondary amine functional group such as DEAE (diethylaminoethyl) is used.

Cation exchange chromatography is conducted with either a strong or a weak cation exchange column containing a sulfonium ion, or with a weak cation exchanger usually having a carboxymethyl (CM) functional group. A counter-ion, often Na^+ maintains electroneutrality.

Agilent offers a wide range of columns for ion exchange:

Table 3: Selection of high resolution ion-exchange columns for charged-based protein separation

Column name	Dimensions	Particle size (µm)	Product number
Agilent Bio MAb	4.6 × 50 mm	3	5190-2403
Agilent Bio MAb	4.6 × 250 mm	5	5190-2407
Agilent Bio MAb	4.6 × 50 mm	5	5190-2408
Agilent Bio MAb	2.1 × 250 mm	5	5190-2411
Agilent Bio MAb	2.1 × 50 mm	5	5190-2412
Agilent Bio SCX	4.6 × 50 mm	3	5190-2423
Agilent Bio SCX	4.6 × 250 mm	5	5190-2427
Agilent Bio SCX	4.6 × 50 mm	5	5190-2428
Agilent Bio WCX	4.6 × 50 mm	3	5190-2443
Agilent Bio WCX	4.6 × 250 mm	5	5190-2447
Agilent Bio WCX	4.6 × 50 mm	5	5190-2448
Agilent Bio SAX	4.6 × 50 mm	3	5190-2463
Agilent Bio SAX	4.6 × 250 mm	5	5190-2467

Column name	Dimensions	Particle size (µm)	Product number
Agilent Bio SAX	4.6 × 50 mm	5	5190-2468
Agilent Bio WAX	4.6 × 50 mm	3	5190-2483
Agilent Bio WAX	4.6 × 250 mm	5	5190-2487
Agilent Bio WAX	4.6 × 50 mm	5	5190-2488

More information can be found at https://www.agilent.com/search/gn/biopharma-hplc-selector.

Buffer Selection

Ion Exchange Chromatography (IEX) Overview:

Typically, low ionic strength buffers are used in ion exchange chromatography to promote the binding of charged proteins to the stationary phase, which carries the opposite charge. Proteins with the same charge as the stationary phase will flow through the column without binding. After the initial binding step, the ion exchange matrix is washed with additional low ionic strength buffer to remove any unbound species.

The bound proteins are then differentially eluted by applying a gradient of increasing salt concentration. As the ionic strength of the mobile phase increases, salt ions compete with the proteins for binding sites on the ion exchange matrix. This competition displaces the bound proteins, allowing them to elute from the column.

Buffer Selection:

For cation exchange chromatography (where the stationary phase is negatively charged), buffers with a pH lower than the protein's pI are typically used. This ensures that the protein is positively charged and can bind to the negatively charged stationary phase. Anionic (negatively charged) buffers are often employed in this scenario. Conversely, for anion exchange chromatography (where the stationary phase is positively charged), buffers with a pH higher than the protein's pI are used, ensuring the protein is negatively charged and can bind to the positively charged stationary phase. Cationic (positively charged) buffers are used in this case.

pH and Buffer Capacity:

A buffer is characterized by its pKa, the negative logarithm of the acid dissociation constant (Ka). The buffer's capacity is highest when the pH is equal to the pKa of the buffer.

If the pH of the buffer is below the protein’s isoelectric point (pI), the protein will carry a net positive charge. If the pH of the buffer is above the protein’s pI, the protein will carry a net negative charge.

Practical Considerations:

When performing salt gradient elution, it is advisable to start at least one pH unit away from the protein's pI to ensure effective separation. Use a buffer concentration sufficient to maintain its buffering capacity and keep the pH constant throughout the procedure, typically between 20 and 50 mM.

Table 4: Buffers for Cation Exchange Chromatography

Name	pKa (25 °C)
Citric Acid	3.13
Lactic Acid	3.86
Succinic Acid	4.21
Acetic Acid	4.75
Methyl Malonic Acid	5.76
MES	6.27
Phosphate	7.20
HEPES	7.56
BICINE	8.33

Table 5: Buffers for Anion Exchange Chromatography

Name	pKa (25 °C)
N-Methyl-Piperazine	4.75
Piperazine	5.33
Bis-Tris	6.48
Bis-Trispropane	6.65 , 9.1
Triethanolamine	7.76
TRIS	8.07
N-Methyldiethanolamine	8.53
Propane 1,3 diamino	8.88
Ethanolamine	9.50
Piperazine	9.73
Propane 1,3 diamino	10.55
Piperidine	11.12

4 Starting with Buffer Advisor

This chapter tells you how to install the Agilent Buffer Advisor, and gives you an introduction to the different parts of the graphical user interface.

Software Compatibility

Table 6: General software requirements

Component	Details
Windows OS	Windows 10 or Windows 11 64 bit, Professional or Enterprise
.NET Framework	.NET 4.8 and above
Web browser	Microsoft Edge (Chromium-based, as shipped with the supported OS version)

Table 7: CDS and driver compatibility

Chromatography Data System	LC & CE instrument drivers
OpenLab CDS 2.8 and above	3.8 and above
OpenLab Chemstation LTS 01.11 Update 4 and above	3.8 and above

Install or Upgrade Buffer Advisor

1 Log in as an administrator.

2 Download from USB-media or Agilent SubscribeNet the AgilentBufferAdvisor.msi file.

3 Double-click on the AgilentBufferAdvisor.msi file and follow prompts on your screen.

After installation, Buffer Advisor software can be found under All Apps > Agilent Technologies > Buffer Advisor.

The software starts in demo mode. You can use all capabilities of the Buffer Advisor software but you cannot create a gradient result timetable and save it. To use all the features of Buffer Advisor, enter the license key number under Help.

Uninstall Buffer Advisor

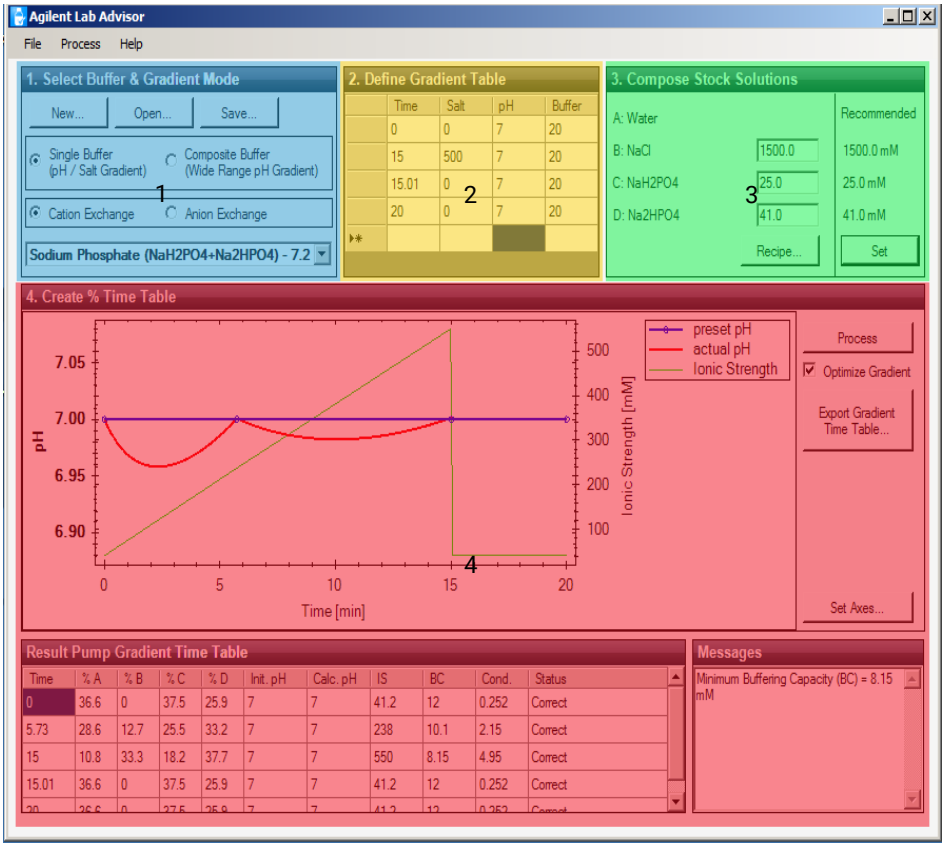
The Buffer Advisor software can be uninstalled via the Windows Control Panel.

1 Log in as an administrator.

2 In the Microsoft Control Panel, open **Programs and Features**.

3 To uninstall, double-click **Agilent Buffer Advisor**.

User Interface Overview



Area 1: Select Buffer and Gradient Solution

Choose between *single buffer mode*, usually applied for salt gradients, and *composite buffer mode* for pH gradients, and whether you want to perform Anion- or Cation-exchange chromatography.

Area 2: Enter Gradient Timetable

Define the gradient parameters such as time, maximum salt concentration (for salt gradients), pH and buffer concentration.

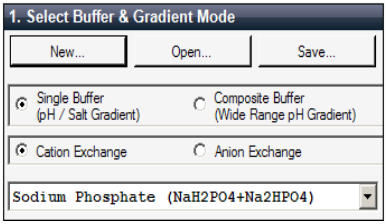
Area 3: Review Composition of Stock Solutions and Adjust

Concentrations for stock solutions are shown, and the recipe button shows amounts of chemical compound to be added to water to prepare the respective stock solutions. You can also enter your own concentrations of stock solutions and the resulting gradient timetable is calculated accordingly.

Area 4: Gradient Display Section: Process Gradient and Review Optimized Gradient with Compensation Steps

When data is entered, the software calculates the percentages of stock solutions for each channel of the quaternary pump. Furthermore, it calculates whether the pH, salt concentration and buffer concentration entered is suitable for the buffer system that was selected. The timetable shows also additional data, such as buffering capacity or ionic strength. A message area gives hints on optimizing the gradient entered in Area 2.

Buffer Selection Area



In the buffer selection area you can select the type of analysis:

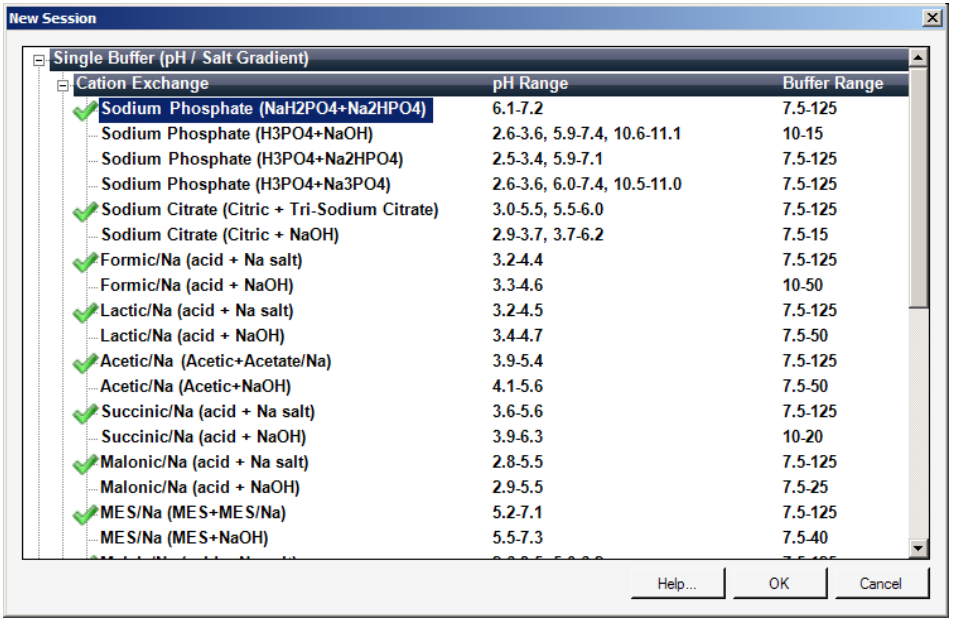
- Single buffer is a buffer consisting of an acidic and basic component. For salt gradients, NaCl can be added in increasing concentrations.
- Composite buffer is a buffer consisting of multiple buffering components. The composite buffer can be used over a wide pH range without compromising the buffer capacity.

Selection of Cation/Anion exchange leads to a dropdown list of buffers for this application. For all buffers, the pKa is displayed, facilitating selection of a buffer related to the analyte's pI, usually near ±0.5 of a buffer's pKa.

Clicking New shows all available buffers in the Agilent Buffer Advisor software, sorted by the above criteria and pKa.

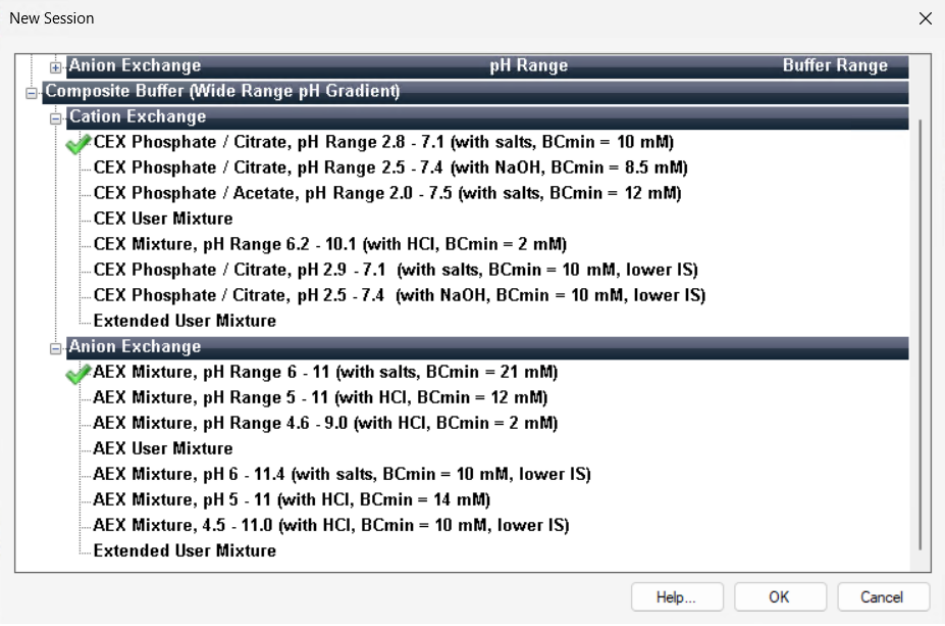
List of Available Buffers

Single Buffers



Composite Buffers

There are several predefined composite buffer mixtures:

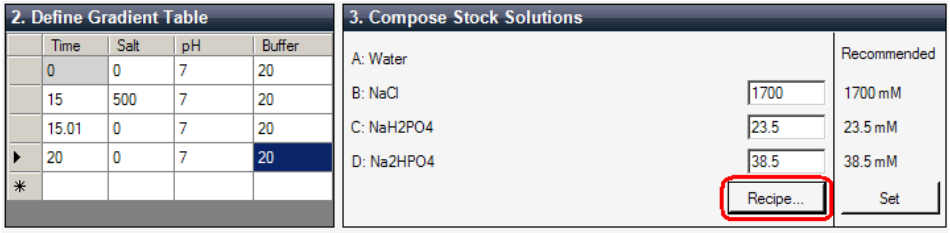


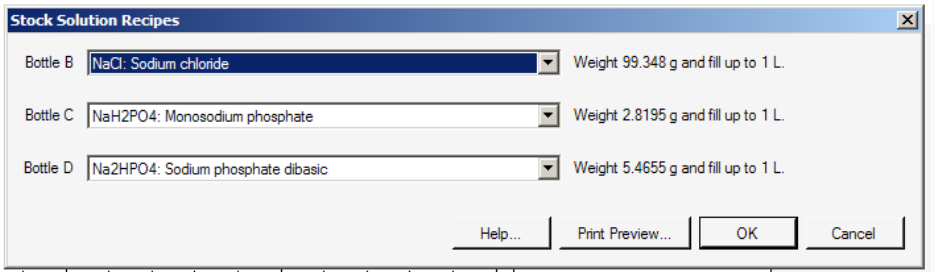
Buffers marked with a green check mark are validated, see Validation Tests and Specifications on page 46.

Depending on the buffer, you select the stock solutions that are displayed on the right-hand side (area 3).

Recipes

Recipe provides the amount of chemicals needed to be weighed in to prepare the stock solutions:


2. Define Gradient Table
Time
Salt
pH
Buffer
0
0
7
20
15
500
7
20
15.01
0
7
20
20
0
7
20
3. Compose Stock Solutions
A: Water
B: NaCl
C: NaH2PO4
D: Na2HPO4
1700
23.5
38.5
Recipe...
Recommended
1700 mM
23.5 mM
38.5 mM
Set


Stock Solution Recipes
Bottle B
NaCl: Sodium chloride
Weight 99.348 g and fill up to 1 L.
Bottle C
NaH2PO4: Monosodium phosphate
Weight 2.8195 g and fill up to 1 L.
Bottle D
Na2HPO4: Sodium phosphate dibasic
Weight 5.4655 g and fill up to 1 L.
Help...
Print Preview...
OK
Cancel

5 Ion Exchange Experiments

This chapter describes detailed workflows for four typical ion exchange experiments.

Salt Gradient with Single Buffer System

A salt gradient experiment is usually designed with a buffer pH around the pKA of that buffer. In this example, phosphate buffer (pKa 7.2) is used with a NaCl salt gradient (final concentration 500 mM).

1 In the **Select Buffer and Gradient Mode** panel, select the **Single Buffer (pH/Salt Gradient)** option.

2 Select the **Cation Exchange** option.

3 Open the dropdown menu and select **Sodium Phosphate (NaH2PO4+Na2HPO4)** from the list of available buffers.

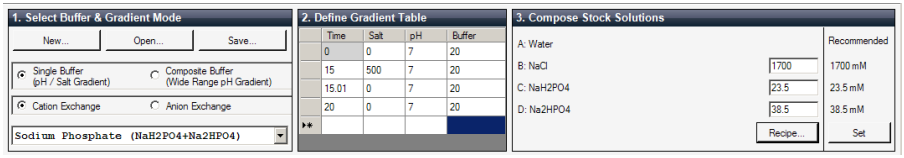
4 In the **Define Gradient Timetable** panel, enter the following gradient timetable parameters:

Time (min)	Salt	pH	Buffer
0	0	7	20
15	500	7	20
15.01	0	7	20
20	0	7	20

This timetable keeps the pH close to the pKa, and creates a salt gradient while keeping the pH constant.

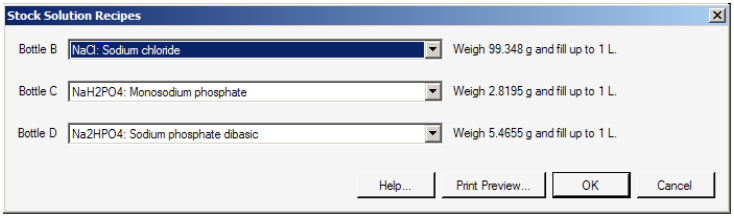
5 To find the best concentration of stock solutions for this experiment, click **Set** under the heading **Recommended** at the right of the **Compose Stock Solutions** panel.

The recommended values above are entered into the fields at the left of the panel.



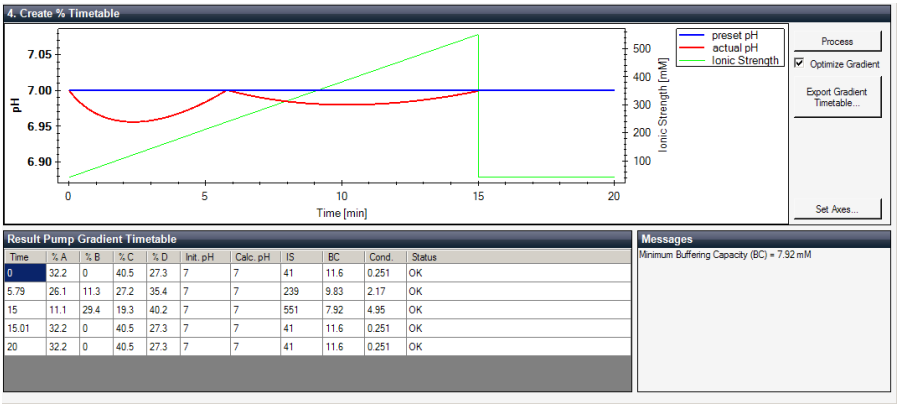
6 Click **Recipe** in the **Compose Stock Solutions** panel.

The **Stock Solution Recipes** dialog box is shown, containing the recipes for the stock solutions (in mg/L or mL/L). You can use these recipes to prepare the stock solutions. Bottle A contains water.



7 Click **OK** to close the **Stock Solution Recipes** dialog box.

An optimized pump gradient timetable is automatically calculated.



Short pH Gradient with Single Buffer System

pH gradients are formed by using buffers consisting of multiple buffer components. Ionic strength must be kept low in order to avoid interference with protein binding. Therefore, only bottles C and D will contain the necessary stock solutions for this experiment. In this example, your protein is stable above its pI (pH 8), therefore an anion exchange buffer is needed. The buffer system is a Tris/HCl buffer (pKa 8.1).

1 In the **Select Buffer and Gradient Mode** panel, select the **Single Buffer (pH/Salt Gradient)** option.

2 Select the **Anion Exchange** option.

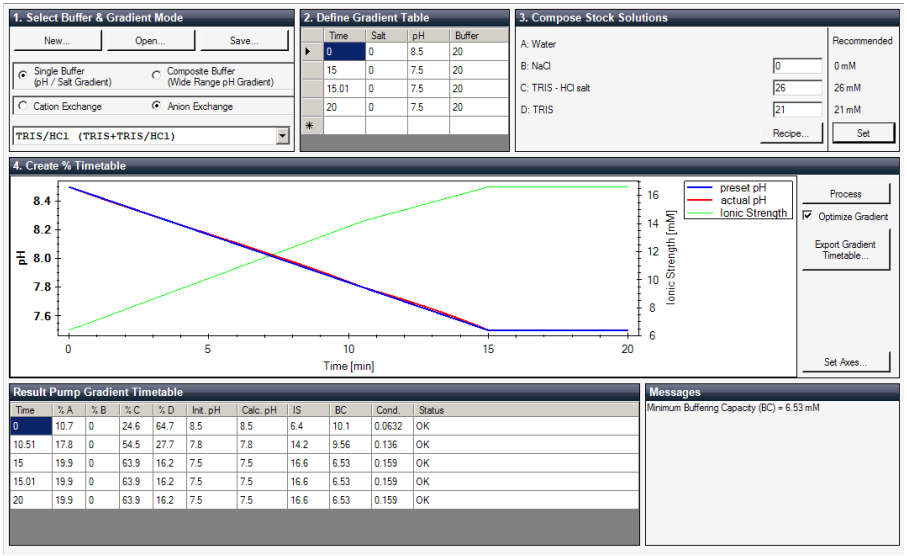
3 Open the dropdown menu and select **TRIS/HCl (TRIS+TRIS/HCl)** from the list of available buffers.

4 In the **Define Gradient Timetable** panel, enter the following gradient timetable parameters:

Time (min)	Salt	pH	Buffer
0	0	8.5	20
15	0	7.5	20
15.01	0	7.5	20
20	0	7.5	20

5 Click **Set** under the heading **Recommended** at the right of the **Compose Stock Solutions** panel and accept the recommended concentrations.

The data is automatically processed and the pump gradient timetable is produced.



You can now set up your stock solutions according to the recipe.

You can also use the single buffer mode for scouting experiments, as described in pH Scouting with Single Buffer System on page 35.

pH Gradient with Multi-Buffer System

pH gradients are formed by using buffers consisting of multiple buffer components. Ionic strength must be kept low in order to avoid interference with protein binding. Therefore, only bottles C and D will contain the necessary stock solutions for this experiment. The bottle containing water can be used for dilution.

Line A	Water
Line B	Not used
Line C	Acidic buffer component
Line D	Basic buffer component

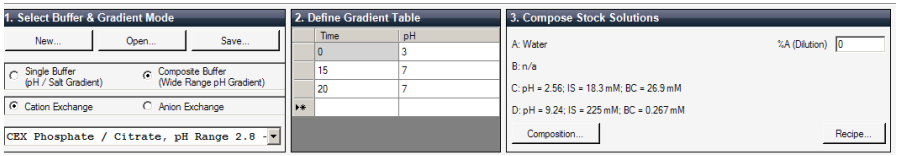
1 In the **Select Buffer and Gradient Mode** panel, select the **Composite Buffer (Wide Range pH Gradient)** option.

2 Select the **Cation Exchange** option.

3 Open the dropdown menu and select **CEX Phosphate/Citrate, pH Range 2.8-7.1 (with salts)** from the list of available buffers.

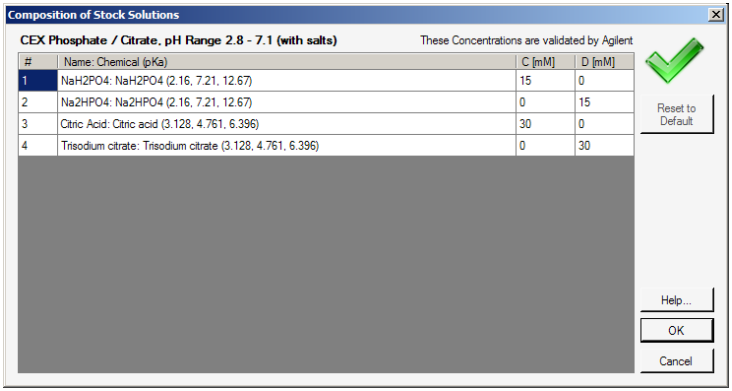
4 In the **Define Gradient Timetable** panel, enter the following gradient timetable parameters:

Time	pH
0	3
15	7
20	7



5 Click **Composition** in the **Compose Stock Solutions** panel to review the composition of the stock solutions.

The **Composition of Stock Solutions** dialog box is shown, containing the suggested compositions of the components.



The green check mark indicates that these concentrations for this multi-buffer system have been validated by Agilent. However, you may change the compositions in the **Composition of Stock Solutions** dialog box and observe the change in pH range in the **Compose Stock Solutions** panel.

6 Click **Recipe** in the **Compose Stock Solutions** panel to display the recipes for each of the selected compositions.

The pump gradient timetable is calculated automatically and displayed.

pH Scouting with Single Buffer System

pH scouting is an approach for screening for optimum resolution of an analyte mixture using a single buffer system at different pH values.

In this example, the buffer system is a phosphate buffer (pKa 7.2) with NaCl (final concentration 500 mM). You can run a salt gradient at pH 6.5, 7 and 7.5.

First, you need to define the boundary conditions with minimum and maximum pH and minimum and maximum salt concentration to find the suitable buffer and salt stock solution that fits for the chosen pH interval.

Table 8: Salt (NaCl) gradient at pH 6.5

Time	Salt	pH	Buffer
0	0	6.5	20
15	500	6.5	20
15.01	0	6.5	20
20	0	6.5	20

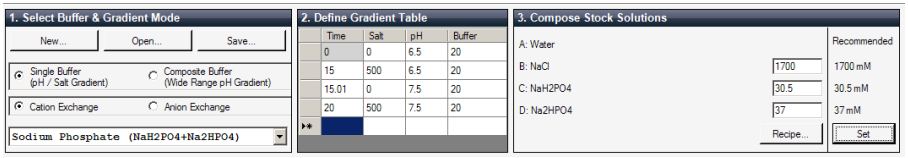
Table 9: Salt (NaCl) gradient at pH 7.0

Time	Salt	pH	Buffer
0	0	7.0	20
15	500	7.0	20
15.1	0	7.0	20
20	0	7.0	20

Table 10: Salt (NaCl) gradient at pH 7.5

Time	Salt	pH	Buffer
0	0	7.5	20
15	500	7.5	20
15.01	0	7.5	20
20	0	7.5	20

1 Set up the timetable as shown in the figure:



Note that the lowest and highest salt concentrations and lowest and highest pH values are combined to define the boundary conditions for the scouting experiment.

2 Click **Set** in the **Recommended** section of the **Compose Stock Solutions** panel.

3 Note down the recommended concentrations given in the **Compose Stock Solutions** panel.

Generate And Export a Gradient Time Table

1 Click **Process** in the **Create % Timetable panel** to calculate a corrected pump gradient timetable.

2 Click **Export Gradient Timetable** to save the corrected gradient timetable in an appropriate file format.

The gradient time table can be saved as *.xml or *.asb file format for import into the LC&CE Instrument Drivers for a quaternary pump.

XML file contains only the exported timetable. ASB file, along with the exported timetable, contains curves of preset and actual pH and ionic strength. Importing the .asb file into OpenLab CDS or ChemStation does not import the pH and ionic strength curves.

Import a Gradient Timetable Into the Quaternary Pump Driver

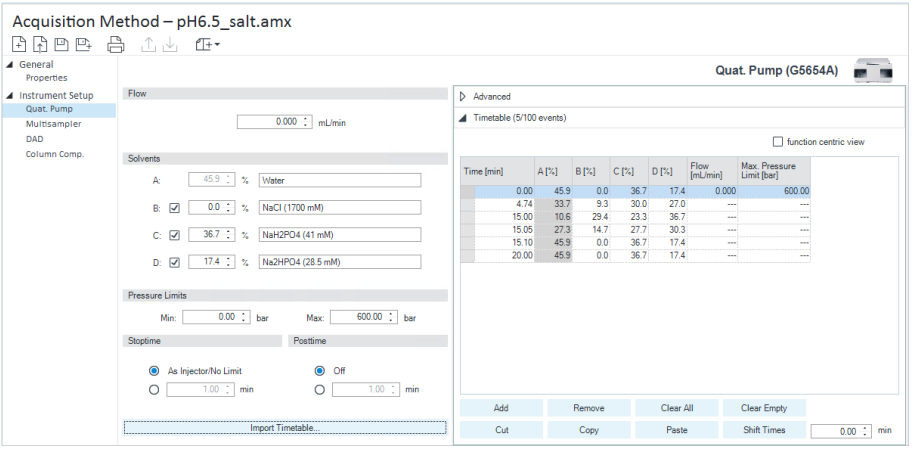
You can import the gradient timetables into the LC&CE Instrument Drivers for quaternary pumps, which are part of the CDS.

1 Go to the OpenLab CDS and set up and save a new acquisition method, for example **pH6.5_salt.amx** .

2 Go to the **Quat Pump** tab on the Method layout and click **Import Timetable**.

3 Select the file that you generated for pH 6.5 in the Buffer Advisor software (in Generate And Export a Gradient Time Table on page 37).

   The timetable is filled automatically with the stored gradient values previously calculated in Buffer Advisor.



4 Save the method.

5 Generate new methods for pH 7.0 and pH 7.5.

   You have now generated three methods in OpenLab CDS with the respective gradient timetables.

Examples of chromatograms obtained upon separation with salt gradient in phosphate buffer at different pH:

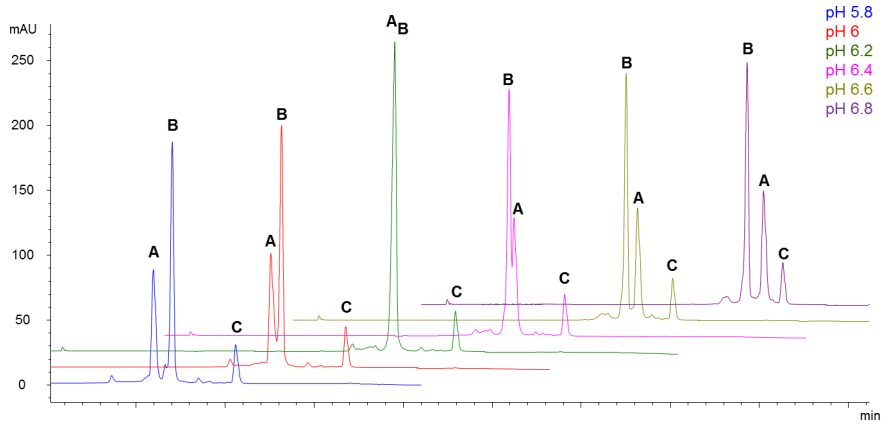


Figure 8: pH scouting experiment using cytochrome C, lysozyme and RNAse.

6 Troubleshooting and Tips

This chapter explains the messages that the Agilent Buffer Advisor provides, and gives you some important information on how to avoid problems.

Messages Shown by Buffer Advisor

Messages in Buffer Advisor software give you hints on how to optimize parameters in the software, such as stock solution concentration or gradient timetable entries for best separation conditions.

Perhaps the buffering capacity (BC) of the buffer is very low, for example, less than 3 mM, and the buffer might not buffer any more, and therefore the entered pH cannot be maintained.

Another example is that a liquid chromatograph's proportioning valve cannot mix one of the solutions precisely if the added percentage of this solution is too low. For best performance, it is recommended always to mix more than 5% per channel.

Here are some parameters that can be modified:

- *Buffer (pH range):* Select a different buffer (system) closer to the pH value you entered in the gradient timetable.
- *Salt concentration (around 500 – 1000 mM):* Ionic strength can influence pH of buffers by lowering buffering capacity. Lower the salt concentration in case of errors and see if the buffering capacity increases.
- *pH:* When you want to keep the same buffer you already selected, change your desired pH in the gradient timetable to ±0.5 of pKa of buffer.
- *Buffer concentration (around 10 – 50 mM):* Increase buffer concentration when your buffering capacity is low or when you get a message that pump mixing is less than 5%.
- *Stock solutions:* Decrease concentration of stock solutions when the 5% warning is shown.

Table 11: List of messages and proposed actions

Message	Action
Calculation process detected database error.	Stay within pH range of selected buffer, lower salt concentration or increase buffer concentration.
Unexpected calculation error ("{0}").	Stay within pH range of selected buffer, lower salt concentration or increase buffer concentration.

Message	Action
Timetable input cannot be calculated with selected buffer. Select different buffer or change timetable.	Select different buffer or change timetable entries to adjust gradient. Adjust parameters as shown above.
Minimum Buffering Capacity (BC) = {0} mM	Only take action when other messages are shown and try to keep it above 3 mM.
Increase mixing per channel > 5% by editing gradient timetable. Choose pH near pKa of buffer, increase buffer concentration or lower salt concentration.	Stock solutions are highly concentrated, leading to a low mixing proportion (less than 5%) of the quaternary pump. Lower stock solutions concentration or increase buffer concentration.
pH difference larger than set threshold {0} detected in "pH Difference" plot.	The pH entered in the gradient table is too far away from the buffer's pKa. Choose pH near pKa, or increase buffer concentration.
Maximum number of lines in result pump gradient timetable reached (maximum is {0} lines). Check pH differences if acceptable.	The pH entered in the gradient table might be too far away from the buffer's pKa so that the corrected gradient timetable contains a lot of mixing steps. Try to stay close to the pKa of the buffer.
Calculation of one or more result pump gradient timetable lines ended with error. Check their status.	Check status line in pump timetable, then adjust parameters.
Stock solution concentration for '{0}' in Bottle {1} exceeded concentration of stock liquid. Maximum concentration is {2} mM.	Final buffer concentration is higher than stock solution concentration. Decrease buffer concentration or increase stock solution concentration.
'{0}' in Bottle {1} may be insoluble in given stock solution concentration. Maximum solubility is expected around {2} mM.	Consider different stock solution concentrations.

Essential Measurement Practices

Storage and Handling

- Handle and store all reagents according to MSDS and the instructions on the label of the individual box.
- Keep all reagent and reagent mixes refrigerated at 4 °C when not in use.
- Allow all reagents and samples to equilibrate to room temperature for 30 minutes before use.
- Use clean bottles and material for buffers and use fresh solvents to avoid microbial growth.

Preparation of Stock Solution Buffers and Salt Solutions

- The buffering substance should have the same charge as the exchanger.
- For the adjustment of pH, the same counter-ion should be used as for elution (e.g. HCl/NaCl)
- Buffer contaminants may produce “ghost” peaks. Use high purity buffer salts.
- Always use a buffering agent that has a pKa within ±0.5 pH units of the operating pH. Otherwise, there is a risk of dramatic pH fluctuation due to the limited buffering capacity of the buffering agent.
- pH varies with the temperature. Tris buffers are particularly temperature sensitive. Adjust pH value after buffers have been equilibrated to the desired running temperature.
- Keep in mind that especially strong bases, for example, NaOH, take CO_2 from air to build carbonate, leading to a change of pH over time.
- Temperature can influence pH. All calibrations and measurements should be performed at the same temperature.
- Note that pH electrodes age and should be replaced on regular basis (i.e. once per year) and/or when pH slope and zero voltage offset are out of recommended range specified by manufacturer.
- Always calibrate your electrode before measurement of pH within the appropriate pH of calibration solutions. Do not use expired calibration solutions and do not reuse them.

- If measuring pH at high ionic strength make sure that your pH electrode is suited for this task.

Mixing Properties of Agilent Infinity III Quaternary Pumps

We have taken great care that the pump properties of the Infinity III Bio LC solutions are taken into account for calculation of optimum conditions of the gradients.

For example, the channels defined for separation must always be A for Water, B for salt and C and D for acidic and basic component.

Be sure to flush the channels properly after use. High concentrations of salt can result in the formation of crystals that can impede the function of the proportioning valve.

- For highest pH precision:
  - Set primary channel in pump driver interface to “A” (accessible via the **Advanced** tab of the Quat. Pump method settings).
  - Make sure that the percentage of acidic and basic solution is always above 5% of the total mobile phase in order to ensure highest gradient precision.

7 Validation Tests and Specifications

Validation Tests and Specifications

In order to guarantee high accuracy of the resulting pH of the mobile phase we tested all indicated buffers under the following conditions:

Instrumentation

Bio-inert quaternary pump (G5611A) connected to a thermostatted column compartment (G1316C with bio-inert heat exchanger G5616-60050), Diode-array detector (G1315C with bio-inert 10 mm path length flow cell G5615-60022) and bio-inert analytical fraction collector (G5664A).

Method for salt gradient mode buffers

Each buffer has been tested at

- three different buffer concentrations (10, 50, 100 mM)
- three different pH values (approximately ± 0.5 pH units distance to published pKa value)
- at least three (up to five) different salt concentrations ranging from 0 to 1000 mM NaCl (5 min salt step gradient)

1 mL fractions were taken over the whole run and pH values were measured offline using an appropriately calibrated pH sensor (Mettler Toledo Inlab micro, Schwerzenbach, Switzerland) connected to a pH meter (Schott handylab pH/LF 12, Mainz, Germany).

All tests were carried out at 23 °C. If the separation in your application takes place at a different temperature, deviations from the set pH can occur.

Method for pH gradient multicomponent buffers

After setting up the two bottles with the indicated constituents and concentrations, a pH step gradient method was performed. Each pH step lasted for 5 min, pH difference between the steps was 0.5 – 1.0 pH units.

1 mL fractions were taken over the whole run and pH values were determined according to the Method for salt gradient mode buffers on page 46.

Stock solutions

Buffer Advisor was used to simulate validation runs in order to identify the minimum number of buffer compound stock solutions necessary to run all validation runs for the corresponding buffer (typically 2 to 3 concentrations of acidic and basic buffer solutions, respectively). Care was taken to maintain acidic and basic channels (C and D) above 5% for highest mixing precision (see Essential Measurement Practices on page 43).

The maximum deviation from set pH in all buffers tested is +/- 0.2 pH units.

NOTE: Some buffers or combination of buffers were not tested and therefore may deviate from the above specifications.

Validated salt gradient buffer list

The following buffers have been tested according to the procedure stated above.

Cation exchange buffers		Anion exchange buffers	
Buffer Name	**pH tested**	**Buffer Name**	**pH tested**
Na-phosphate	6.2, 7.0, 7.7	**Bis-Tris**	6.0, 6.5, 7.0
Na-citrate	4.3, 4.8, 5.3, 5.9, 6.4, 6.9	**Triethanolamine**	7.3, 7.8, 8.3
Formic acid	3.2, 3.7, 4.2	**Tris**	7.6, 8.2, 8.6
Lactic acid	3.4, 3.9, 4,4	**Diethanolamine**	8.4, 8.9, 9.4
Acetic acid	4.3, 4.8, 5.3	**Ammonia**	8.6, 9.1, 9.6
Succinic acid	3.6, 4.3, 4.9, 5.6	**Ethanolamine**	9.0, 9.5, 10.0
Malonic acid	5.2, 5.7, 6.2		
MES	5.6, 6.1, 6.6		
Maleic acid	5.8, 6.3, 6.8		
MOPS	6.7, 7.2, 7.7		
HEPES	7.1, 7.6, 8.1		
TAPS	8.1, 8.6, 9.1		
Na-bicarbonate	9.3, 9.8, 10.2		

Validated multi-component buffer list

Multi-component buffer CEX Phosphate/Citrate, pH Range 3 – 7 (with salts) was tested at following concentrations and conditions:

	Bottle C	Bottle D
Na_2HPO_4		15 mM
NaH_2PO_4	15mM	
citric acid	30 mM	
trisodium citrate		30 mM
pH calculated	2.55	9.25
pH measured	2.50	9.04

A pH gradient was run from pH 3.0 to pH 7.5.

Multi-component buffer AEX, pH Range 6 – 11 (with salts) was tested at following concentrations and conditions:

	Bottle C	Bottle D
Bis-Tris		60 mM
Bis-Tris-HCl salt	60 mM	
Tris		30 mM
Tris-HCl salt	30 mM	
Ethanolamine		40 mM
Ethanolamine-HCl salt	40 mM	
Piperidine		35 mM
Piperidine-HCl salt	35 mM	
pH calculated	3.94	11.79
pH measured	3.97	11.80

A pH gradient was run from pH 6.0 to pH 11.0.

In This Book

This manual describes the Agilent Buffer Advisor software and its use. It gives background information on ion exchange chromatography, and describes typical workflows for defining stock buffer solutions for ion exchange experiments.

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