



CHEMICAL SOLUTIONS, Inc.

3392 State Hwy 8, South New Berlin, NY 13843

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www.chemicalsolutionsinc.com

(607) 859-2706 fax (607) 859-2917

HPLC PERFORMANCE QUALIFICATION SYSTEM

OPERATING INSTRUCTIONS

Rev. 1.08



**HPLC PERFORMANCE QUALIFICATION SYSTEM
OPERATING INSTRUCTIONS**

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PQ KIT PRODUCT DESCRIPTION:

The HPLC Performance Qualification System is a unique product designed to quickly and easily qualify an isocratic or gradient HPLC system for performance levels, while maintaining full NIST-Traceability, and meeting cGMP requirements.

The PQ Kit contains a series of certified solutions, along with a special-purpose reversed-phase HPLC column and an Excel™ - based software template, all of which are designed to work in concert to produce a fully-documented, regulatory compliant instrument qualification within hours.

By using a dedicated, premium column with a simple mobile phase composition tightly controlled through a System Suitability Test, accurate and reliable inter-laboratory test results anywhere in the world are assured.

The various test protocols and materials can be readily incorporated into your laboratory SOP's, and will be found to be invaluable during method development and method validation (especially method transfer), as well as for general method and instrument troubleshooting and maintenance.

The PQ Kit has materials and protocols to test and document for the following HPLC Performance Qualification tests:

- Pump Flow Accuracy at up to 3 flow rates
- Column Oven Temperature Accuracy at up to 3 temperatures
- HPLC Extra-Column System Volume (volume variance)
- HPLC Gradient Dwell (delay) Volume
- HPLC Gradient Delivery Accuracy and Profiles
- HPLC Pump Drift (Retention Time Precision)
- Detector Wavelength Accuracy at up to 14 wavelengths from 241-641 nm (Primary Standard)
- Detector Wavelength Verification @ 205 nm and 273 nm (Caffeine Secondary Standard)
- Detector Short-Term Dynamic Noise
- Detector Sensitivity and Caffeine LOD @ 273 nm (Normalized for Time and Efficiency)
- Autosampler Injection Precision and Drift
- Autosampler Injector % Carryover
- Autosampler Volume Linearity and Precision from 1-50 µL (can be extended to 100µL)
- HPLC System Linearity and Precision over 3 orders (100% to 0.1%) within an absorbance range suitable for most detectors

The PQ Kit offers "System" Performance Qualification tests. These tests are designed to be used to confirm suitable instrument operation after routine preventative maintenance of individual components, after major repairs or moving the location of an instrument, and on a routine semi-annual basis to periodically document proper instrument performance.

Most of the above tests are independent. Thus, only those test protocols of interest to the user need be performed. Typical Performance Qualification times for an isocratic system will be 3-5 hours, depending upon the tests chosen. Gradient qualification requires approximately 1 hour per solvent pair. Except for the Primary Standard Wavelength Accuracy test, all test protocols are fully automated, and are run as a normal HPLC sequence without user intervention.

The PQ Kit is economical, with no restrictions on the number of HPLC's that can be qualified using a single kit.

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Item. No.	Description:	
PQCC0101	HPLC Performance Qualification Kit with PQ Test Column	
	The following components are contained within the kit:	
	PQCOLA01	PQ Test Column , with Certificate
	PQAHOX01	Wavelength Calibration Solution, NIST-Traceable
	PQARTM01	Resolution Test Mixture
	PQAL0101	Linearity Solution L1
	PQAL0201	Linearity Solution L2
	PQAL0301	Linearity Solution L3
	PQAL0401	Linearity Solution L4
	PQAL0501	Linearity Solution L5
	PQAL0601	Linearity Solution L6
	PQAGVS01	Gradient Visualization Solution
	PQACDR01	Excel Template with PDF instructions (CD-Rom)
	PQACFT01	Certificate of Analysis with statement of NIST-Traceability for quantitative solutions provided above.
Replacement Components:		
PQNC0101	HPLC Performance Qualification Kit - Replacement Solutions only	
PQCOLA01	PQ Test Column	
PQAFKT01	HPLC Cell Filling Syringe and Fittings Kit	

For technical support, contact:

Chemical Solutions, Inc.
3392 State Hwy 8
South New Berlin, NY 13843

(607) 859-2706 fax (607) 859-2917

also, visit our website at www.chemicalsolutionsinc.com**IMPORTANT SAFETY INFORMATION****WARNING:****THE WAVELENGTH CALIBRATION SOLUTION CONTAINS 10% PERCHLORIC ACID. PERCHLORIC ACID IS A STRONG OXIDIZING AGENT. USE APPROPRIATE EYE AND SKIN PROTECTION. DISCARD EXCESS SOLUTION BY POURING INTO A LARGE QUANTITY OF WATER, DILUTING FURTHER, THEN DISCARDING IN AN APPROPRIATE MANNER. CONCENTRATED PERCHLORIC ACID CAN CAUSE FIRE WHEN IN CONTACT WITH ORGANIC MATERIAL.**



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To prepare for a Performance Qualification of your HPLC, you should print and/or view these other documents, contained in your CD-Rom, containing more in-depth instructions.

- PQ Excel Template (regular and Demo versions containing sample data)
- Excel Operating Instructions
- PQ Kit Quick Start
- PQ Kit Summary Form (can be used to summarize all test results on a single page)

The PQ process is very similar to running a regular validated method assay for a number of pre-made, certified samples. You should always follow your own internal laboratory SOP's regarding documentation and procedures.

A Performance Qualification assumes an instrument in good operating condition. Perform and document any routine service or maintenance of the HPLC, including seals, check valves, rotors, lamps, etc. prior to commencing the PQ. In many companies, this is performed by factory service personnel, and constitutes the Operational Qualification (OQ) of the HPLC.

STEPS FOR A SUCCESSFUL HPLC PERFORMANCE QUALIFICATION:

- **Prepare the HPLC** - Perform any Maintenance, lamps, seals, etc.
- Prepare the **Mobile Phase**. If a Gradient Qualification is planned, reserve ca. 500 mL of mobile phase to spike with the Gradient Visualization Solution (GVS).

Manual PQ Operations:

- Qualify the pump **Flow Rate Accuracy**.
- Qualify the **Column Oven** temperature. The PQ Tests do not require a column oven.
- Perform the **Wavelength Qualification Test**

Automated PQ Operations:

- Create the instrument **Methods**.
- Create the **Injection Sequence**, based on the tests you decide to perform.
- Prepare the **Injection Vials**, based on the sequence.
- **Run the PQ tests** as a single, fully automated sequence.
- Enter the results into the **PQ Kit Excel Template**. The template automatically calculates and graphs all data.
- **Print and Review the final results**, confirming accurate integration and data entry. The template references all results to a single front page for review and sign off.

You are done! Once completed, your HPLC is fully Performance Qualified against NIST-Traceable standards. The wealth of data provided under standardized conditions, allows you to easily monitor changes in the performance of your HPLC over time, and to compare the sensitivity and performance another HPLC anywhere in the world. This is of great utility in troubleshooting method transfers. The PQ Tests also can function as an excellent training documentation tool for new laboratory personnel.



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I. INTRODUCTION - PRINCIPLES OF OPERATION:

The tests described are intended to document and qualify various performance criteria of an HPLC system. Typically, the circumstances under which such tests must be performed will be described by internal company Standard Operating Procedures (SOP's). As experience is gained with the individual test protocols, many companies have found it profitable to incorporate these tests into future revisions of their relevant SOP's.

Performance Qualification Tests are typically performed on HPLC systems under the following circumstances:

- After major service, such as the routine Preventative Maintenance procedures, typically involving pump/injector rebuilding and servicing of other components
- On a routine basis, in between major service schedules
- After moving an HPLC from one location to another
- To troubleshoot either method or instrument problems, to document and isolate or rule out instrument problems
- As universal test when the need arises to compare instrument performance at other laboratories worldwide

IQ/OQ/PQ - What are the Differences?

Each laboratory will have their own specific written instructions detailing what constitutes the Installation (IQ), Operational (OQ) and Performance Qualifications (PQ) procedures. In general however, the following is a fairly common division among these tasks.

The Installation Qualification is the documented verification that the software and hardware installation meet the instrument manufacturer's requirements, and that the instrumentation has been properly installed as specified. In addition to installation itself, part of the IQ procedure is to bring the instrument into the laboratory documentation system. This includes bringing the instrument into the laboratory database (if one is used), documenting where the instrument manuals will be stored, setting up an instrument logbook, and generally assuring, and documenting, that the instrument is properly installed and ready for operation.

The Operation Qualification is the next logical step, and is designed to test and document that the system operates as intended throughout its anticipated operating range. Many laboratories take the opportunity with the OQ to "put the instrument through its paces", i.e., to document the instrument performance at the limits of its intended performance range. The OQ is typically performed on the individual instrument components. For example, pumps may be leak checked at 414 bar (6,000psi), even though they would never approach this in normal operation. Detector noise and performance may be documented using the manufacturer's internal diagnostic procedures. Most modern instruments contain most or all of the internal diagnostic procedures required to achieve a thorough OQ, but if not, procedures need to be written to properly test the performance characteristics of the instrument components. The OQ is often performed by the manufacturer as the final step in the installation of a new HPLC or component.

The final qualification procedure, and the one that will be performed routinely at set times, or after minor maintenance is the Performance Qualification.

By design, Performance Qualification tests for an HPLC test the functionality of HPLC as a complete, working System. For example, a test of autosampler delivery precision is dependent upon proper operation of both the pump and detector. When a pump is rebuilt by a service technician, specific factory tests are performed to measure pressure leak-down and similar specific performance measures. Performance Qualification tests are designed to complement, rather than replace, these types of component tests. Once each of the HPLC components are inspected and qualified by the service technician, the Performance Tests document the operation of the entire HPLC system in the laboratory under realistic test conditions.

HPLC System Performance Qualification tests should be performed frequently, on a routine basis, to document system



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performance under uniform test conditions, and to compare the instrument performance to historical values. Once every 6 months is a common schedule for many companies.

Performance Qualification testing has typically been a hit-or-miss affair, with every laboratory having different procedures, sometimes even within a single company. The PQ Kit was designed specifically to provide uniform, high quality test protocols and materials, which can be used to document HPLC performance levels, with confidence.

Traceability to recognized standard reference materials is the cornerstone of a solid, defensible laboratory qualification program. The Wavelength Calibration Solution is identical in composition to NIST Standard Reference Material 2034, and is directly traceable to authentic NIST standards during manufacture. All other solutions are manufactured with traceability to NIST standard materials.



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Overview of the Operating Instructions:

The Operating Instructions are intended to provide an overview and “quick start” to using the methods and materials contained within the PQ Kit. As experience is gained, it will be found that the required methods and injection sequences will have been pre-written, and can be stored and re-used along with these instructions, just as for any routine method in your laboratory.

The following sections of the operating instructions describe how to set up your HPLC using the provided test kit materials. The provided software is certified to produce accurate results with Excel™ 2000 or newer (older versions will work, but are not guaranteed to format consistently). The software contains instructions and numerous drop-down selections to aid data entry, and is password protected to avoid accidental changes. Once entered and saved, the results are printed normally, where they can be reviewed and signed, and entered into the normal laboratory instrument qualification records. The various tests are not dependent upon the included software for successful completion. Some laboratories may prefer to enter the various test results manually, or to maintain them within their own LIMS or other system.

Materials and Methods are described in separate sections. The user is required to make sufficient volume of mobile phase for the various tests, but all other solutions are provided, requiring only that they be vialled.

Detector Wavelength Accuracy is a manual test, and may be performed either before or after the other Test Protocols.

It is assumed that the PQ Tests are being performed on an HPLC in good condition, which has an accurate flow rate at 1.0 mL/minute. Documented accuracy at this flow rate is required, since other tests (such as Gradient Dwell Volume and Extra Column Volume, use the flow rate for calculation of these parameters. In addition, space is provided for calibration at 2 additional flow rates, chosen by the user. Common qualification flow rates for a conventional HPLC would be 0.5, 1.0 and 2.0 mL/min, as the minimum and maximum flow rates the instrument is likely to be used at.

The flow rates are most easily qualified using a calibrated flow meter. If one is available in the laboratory, simply qualify the pump at the 3 flow rates, following the instructions provided with the meter. Space is provided in the software to enter the flow meter serial number and calibration date. If a meter is not available, simply collect the eluent into clean, dry volumetric flasks of appropriate volume, timing the fill times with a calibrated stopwatch, as described in the detail in a later section.

Column Oven Temperature qualification is another preparatory test, which is normally performed independently of the general PQ tests. Space is provided in the software for calibration at up to 3 different temperatures. The calibration temperatures chosen will vary depending upon the design of the oven, and its most common usage. If the oven is capable, sub-ambient temperatures may also be chosen.

After flow qualification, the Resolution Test Mixture is injected into a stable HPLC system (confirmed by prior blank injections), to ensure proper mobile phase preparation and adequate column performance, as required to produce reliable, consistent test data. The mobile phase may be adjusted to meet System Suitability requirements. The mobile phase is chemically stable for 18 months.

A master injection sequence PQ01 is listed, showing a complete isocratic and gradient HPLC qualification. The user should generate their own sequences including only the tests they wish to perform, and corresponding methods as required by their instrumentation.

It is a responsibility of the user to initially run test chromatograms, and to determine suitable integration parameters and other method details as required by their particular instrumentation. Due to the consistency of the test columns and conditions, once set, these integration parameters should rarely have to be changed during future runs.

In various sections, reasonable Acceptance Criteria are suggested for the individual test results. The user should set whatever Acceptance Criteria they feel are reasonable or justified for each of the various individual test protocols, and to match their internal SOP's.



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II. QUICK-START OVERVIEW:

The basic procedures needed to perform a Performance Qualification of your HPLC are:

1. Primary Standard Wavelength Accuracy Solution:

- The qualification of detector wavelength accuracy with the primary standard Holmium Oxide requires that the flow cell be filled manually via syringe. Since this test is not part of the automated injection sequence, it may be performed separately either before or after the HPLC runs. It is recommended that it be performed first, since failure of the Wavelength Accuracy will invalidate all subsequent tests.
- Fill the flow cell using the HPLC Cell Filling Kit. Obtain spectra either manually (for VWD) or with software (for DAD), and enter the results into the appropriate areas of the provided software. The found absorbance maxima will automatically be compared against the official NIST values, and regression plots of the found vs true wavelengths automatically performed.
- Determine a reasonable Acceptance Criteria for Wavelength Accuracy. In the absence of instrument specs or internal SOP's, $\pm 1/2$ the detector bandwidth is recommended. Typical specifications will be $\leq \pm 2\text{nm}$ for newer detectors, and $\leq \pm 3\text{nm}$ for older units.
- While calibration against the Primary Standard is recommended, detector wavelength accuracy may also be qualified using one of the Caffeine solutions provided in the kit. Caffeine has moderately sharp maxima at 205 nm and 273 nm .

2. Prepare the mobile phase:

- Prepare the Isocratic Mobile phase
- For Gradient Qualification, reserved about a 500 mL aliquot to spike with the Gradient Visualization Solution (GVS @ 6 mL/L).
- NOTE: THE MOBILE PHASE IS STABLE FOR 60 DAYS, WHEN KEPT IN A SEALED CONTAINER TO PREVENT EVAPORATION. It can be prepared in bulk for multiple instrument qualifications. The mobile phase may be adjusted to meet System Suitability.

3. Prepare the HPLC and PQ Test Column:

- Flush all fluid lines with the required mobile phases
- For Gradient Qualification, use the "B" pump/selenoid line for the spiked (B*) mobile phase
- Note that the results of the Gradient Test depend upon a fully-flushed, constant composition of mp in the B* fluid circuit. Flush thoroughly. If in doubt, program 2 runs into the final sequence, using the 2nd run for the test results.
- Be sure the HPLC is fully warmed and equilibrated as per manufacturer's directions.
- The PQ Test column is shipped, and should be stored, in the method mobile phase. Since the column is used only for test purposes, it should never become contaminated, and should require only a minimal 10-15 minute equilibration prior to starting the sequence. If it ever requires cleaning, flush for 15-30 minutes with 50:50 acetonitrile:water.



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4. Prepare the HPLC Methods:

- Write the Base Method PQ10 to suit your HPLC. See Methods section for details. This is a simple isocratic method, 10 μ L injection, 273 nm, 1 mL/min. Run time needs to be sufficient only to integrate the Caffeine peak used for most of the tests, typically 5-6 minutes. Some test runs may be required to set proper integration parameters the first time the method is developed.
- The RTM10 method is identical to method PQ10 developed above, except it runs for 10 minutes.
- If Autosampler Volume Linearity is to be performed, create 4 other new methods, all identical to the PQ10 base method, except for the injection volumes to be tested.
- For Gradient Qualification, write a method for each solvent pair to be tested, using 5 minutes steps at 50% increments, as detailed in the Methods section.

5. Prepare the Injection Sequence:

- Determine which Test Protocols you are going to perform. Write an injection sequence suitable for your instrument, depending upon whether you are using multiple injections per vial, or single vials.

6. Fill the Injection Vials:

- Fill the required number of injection vials with the appropriate solutions. Be careful not to cross-contaminate the parent solutions, and use normal good laboratory procedures.
- For Blanks use the Mobile Phase. Note that the Gradient Dwell Volume requires injection of a blank of the GVS-spiked B* mobile phase. Other gradient tests use the normal unspiked mobile phase.
- Depending upon whether you prefer to inject multiple times from a single vial, or perform only 1 injection per vial, prepare the appropriate number of vials (see Example Sequence in next section).

7. Start the Sequence:

Using triplicate injections for each point, with 6 min run times, total sequence run times will be on the order of:

- For an isocratic qualification, without Injection Volume Linearity, about 3.5 hours
- Injection Volume Linearity - an additional 1.5 hours
- Gradient Qualification - about 1 hour per solvent pair (3 hours total for Quaternary system).

8. Analyze the Results:

- Enter the Certificate of Analysis solution concentrations, instrument details, and results into the PQ Kit Excel[™] template- print final qualification forms - about 15 minutes



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III. MATERIALS:

A. Software:

1. The PQ Kit contains dedicated software in the form of a protected Microsoft Excel[™] 2000 (or higher) spreadsheet template for entry and calculation of all test results. The program is included on the CD-Rom accompanying the kit, along with Adobe Acrobat files of all instructions and written material. The user should copy the spreadsheet to their hard drive, and incorporate the program into their laboratory data systems in a manner consistent with their compliance with CFR 21 Chapter 11 or other requirements.
2. The user is not required to utilize this software to perform the tests. However, the provided software is certified to produce numerically correct results for each of the tests when operated as per these instructions. The software is specifically designed to assist the user in meeting typical cGMP documentation requirements.
3. The software is designed to work with Excel[™] 2000 or higher versions. Compatibility with earlier versions is not guaranteed.
4. The provided software is password protected. If company policies require that the software be unlocked to central distribution on a server, or if customization to suit particular company needs is required, please call Chemical Solutions, Inc.
5. Many of the tests utilize linear regression and other statistical tools contained within Excel[™]. It is necessary for the user to have loaded the "Analysis ToolPak" under the "Tools .. Add-Ins" menu, which comes standard with all versions of Excel[™] 2000, and higher.
6. The software is copyrighted by Chemical Solutions, Inc. However, it may be freely copied and made centrally available by the end user for support of the PQ Kit within his or her company. The software may not be re-sold or otherwise distributed.
7. The revision number is contained in the upper left area of the template. The software is continually updated, so the user should check the Chemical Solutions website www.chemicalsolutionsinc.com for the latest available revision. The website also contains other important information, such as the CoA concentrations for the various kit lot numbers.

B. Required Reagents for Mobile Phase Preparation

1. Purified Water, USP or better
2. Acetonitrile, HPLC grade
3. Glacial Acetic Acid, ACS Reagent grade or better



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IV. PROCEDURES:

A. Introduction

For clarity, the Wavelength Qualification, which is a manual operation involving filling the flow cell with the Wavelength Calibration Solution, is described in a later section. It may be performed either before or after the other qualification tests, which use the autosampler and regular injections. In practice, all Test Protocols must pass for a successful PQ, so the order of testing is not important.

The following example injection sequence summarizes a master-list of the various tests available for the Performance Qualification of an HPLC system using the PQ Kit. Modify the sequence as required to omit tests not used, such as the Gradient tests.

The test protocols may be performed in any order, except for System Suitability. The liberal use of fresh blanks from new vials is encouraged to minimize the risk of carryover. Sequence PQ01 is used as an example, but is not meant to be limiting.

Sequence PQ01 is written assuming that your autosampler (and internal policies) allow for multiple injections from a single vial. If single vial injections are to be used, change the sequence as required to reflect this.

Note that the injector carryover test may be performed with or without a wash vial, if your autosampler has that option. Document which method is used.

See the next section for detailed discussion of Wavelength Calibration procedures.

B. Initial Instrument Qualifications:

1. Flow Accuracy:

Provisions are included in the software and Summary Form to measure and record the results for up to 3 chosen flow rates. This may be accomplished either manually using volumetric flasks and a calibrated stopwatch, or by using a calibrated flow meter. You should use the mobile phase and PQ column for this test, for flow rates of up to 2 mL/min. For higher flow rates, remove the column to prevent damage from excessive pressures. Use a restriction capillary if necessary, to produce sufficient backpressure for the check valves to operate properly, if required.

If you use a flow meter, simply record its serial number and calibration date, etc. in the spaces provided in the template or form. Follow the flow meter instructions for its operation.

If you perform the flow rate check manually, select clean, dry class A volumetric flasks as appropriate for each flow rate. The flow rate can be calculated as follows:

$$\text{Flow Rate mL / min} = \frac{\text{Flask Volume mL}}{\text{Minutes to Fill (decimal)}}$$



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To convert min:sec to decimal minutes, simply divide the seconds by 60, and add to the minutes. For example, if you are qualifying the pump at 2 mL/min, using a 10 mL volumetric flask, and it takes 4 minutes and 57 seconds to fill the flask, the flow rate would be:

$$\text{Decimal Minutes} = 4 \text{ min} + (57 \text{ seconds}/60 \text{ sec}/\text{min}) = 4 \text{ min} + 0.95 \text{ min} = 4.95 \text{ min}$$

and the actual flow rate would be:

$$\text{Flow} = 10.0 \text{ mL} / 4.95 \text{ min} = 2.02 \text{ mL}/\text{min}$$

In terms of % Accuracy, this would be:

$$\% \text{ Flow Accuracy} = 2.02 \text{ mL}/\text{min} / 2.00 \text{ mL}/\text{min} (\text{nominal}) * 100 = 101.0\%$$

Consult your instrument manual for appropriate flow accuracy specifications. Many laboratories will typically specify ± 5% accuracy for flow rates.

Note that the Gradient Dwell Volume and Extra-Column Volume tests utilize the results of the true flow rate at a nominal setting of 1.0 mL/min. Thus, it is necessary to enter the qualification results for this flow rate. The other flow rates are optional, and may be selected by the user, or left blank if only a single flow rate is to be tested.

For flow rates of 0.5 - 2.0 mL/min, the column should be attached in order to produce sufficient back pressure to operate the check valves properly. However, if a high flow rate, such as 5 mL/min is to be qualified, it is essential that the column NOT be connected, as the column could be damaged by the high back pressures. Normally, the narrow connecting tubing alone will provide sufficient back pressure at these high flow rates. If not, add a length of narrow bore tubing via a union to produce the necessary back pressure at higher flow rates. Recommended volumetric flask sizes are as follows:

Recommended Volumetric Flask Sizes for Pump Flow Qualification:		
Nominal Flow Rate:	Recommended Vol. Flask	Nominal Fill Time:
0.5 mL/min	5 mL	10 minutes
1.0 mL/min	5 mL	5 minutes
2.0 mL/min	10 mL	5 minutes
<i>**5.0 mL/min</i>	<i>25 mL</i>	<i>5 minutes</i>

** Do not perform this flow rate with the column attached!! The pressure will be excessive and damage to the column will occur.

Finally, note that the National Institute of Standards and Technology (NIST) provides a phone number for stopwatch calibration. The phone number is 303-499-7111. A 1 second repeating tone is provided, that can be used to qualify your stopwatch. Follow your companies internal SOP's regarding qualification procedures.



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2. Column Oven Temperature Accuracy:

Provisions are made in the software and accompanying documentation to record up to 3 temperatures for a column oven. Unused temperature cells should be left blank, if fewer than 3 temperatures are to be qualified.

If your company has written SOP's describing column oven qualifications, follow those procedures. Most SOP's call for the use of a calibrated thermocouple or thermometer, which is inserted into the column oven cavity in such a manner as to mimic the column placement. Air drafts should be prevented by sealing any openings with foam or other material. Do not allow the thermocouple to directly contact any of the heated surfaces. Temperature equilibration is typically documented by recording duplicate temperature readings several minutes apart. The two temperatures should agree by better than 1/2 of the temperature accuracy specification.

Consult your instrument manual for appropriate accuracy specifications. Many laboratories will typically specify a temperature accuracy of $\pm 5^{\circ}\text{C}$.

C. Instrument Preparation:

1. Prepare Mobile Phase, according to the Solutions Preparation section of these instructions. Note that only a single common mobile phase is used throughout. The Gradient Test simply spike aliquots of this same mobile phase with the Gradient Visualization Solution (no volume correction is needed). Thus, it will be most efficient to prepare larger volumes of mobile phase at a single session, then split off what is required for gradient instruments, or for multiple HPLC qualifications.

Note that the mobile phase is chemically stable for up to 18 months at ambient laboratory temperatures from the date of preparation, provided evaporation is prevented. The mobile phase can be adjusted to meet system suitability requirements. Laboratories with many instruments to qualify may find it useful to prepare large quantities, and to store the mobile phase in 4L brown glass solvent jugs. The GVS-spiked mobile phase may also be stored, but the bottle contents should be clearly labeled to prevent confusion with unspiked mobile phase.

2. Setup HPLC using methods from the Methods section of these instructions, as appropriate for the tests to be performed.
3. Attach the PQ Kit Test Column, and equilibrate with the Isocratic Mobile Phase. Note that the column is shipped in the method mobile phase, so that only minimal column equilibration is required. The column should be stored in the method mobile phase when done.

Prior to initiating any of the tests, ensure that the instrument is fully stabilized with steady flow, with the required minimum detector warm-up times as suggested by the manufacturer being followed.

4. The PQ Kit is operated at ambient column temperatures, which is defined for test purposes as between 15°C - 27°C (60°F - 80°F). The PQ Test column may be used in a column oven within this temperature range, but a column oven is not required.



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D. Test Protocol Summary and Master Injection Sequence:

1. Vial Preparation and Injection Sequence:

An injection sequence should be prepared as appropriate for your laboratory practices and the instrument to be qualified. For example, some laboratories have written policies that separate injection vials must be used for each injection, while others permit multiple injections from a single vial.

Tests in our own laboratories have shown that due to the short run times (4-5 minutes) of the Caffeine standard, multiple injections from a single vial can be used without the risk of evaporation.

Preparing the Vials:

The following Table summarizes the injection vials you should prepare, to perform the full suite of tests contained in Sequence PQ01, using multiple, or single injections per vial. Prepare injection vials appropriate for your instrument, using the Isocratic Mobile Phase A, and the solutions from the PQ Kit materials. Be careful to avoid cross-contamination of the bottles, and to re-cap them to prevent evaporation. If a Gradient qualification is to be performed, fill a vial with the GVS-Spiked mobile phase "B*", to inject as the blank for the first gradient pair to be qualified.

Sample Vials Required to Perform Sequence PQ01 - All Test Protocols		
PQ Kit Sample	For Multiple Injections per Vial	For Single Injection per Vial
Mobile Phase A Blank	6 vials	12 vials
Rs Test Mixture	1 vial	1 vial
L3 Linearity Solution	3 vials	28 vials
L1 thru L6 Linearity Solutions	1 vial each	3 vials each
Mobile Phase Blank from GVS-Spiked B* mobile phase (only if gradient HPLC is tested)	1 vial	1 vial



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Sequence PQ01: Example Injection Sequence PQ01 Using Multiple Injections from Single Vials					
Test Protocol:	Vial Position	Sample Name	Method	No. Injections	Inj. Vol
System Suitability System EC Volume Detector Noise	1	Mobile Phase A Blank	RTM10	2	10 µL
	2	Resolution Test Mixture (RTM)		1	
Injection Precision Pump Flow Precision/Drift	3	L3	PQ10 ^a	10	
Cleansing Blank	4	Mobile Phase A Blank		2	
Detector Linearity System Sensitivity/ Caffeine LOD	5	L1		3	
	6	L2		3	
	7	L3		3	
	8	L4		3	
	9	L5		3	
	10	L6		3	
Injector % Carryover	11	Mobile Phase A Blank		3	
Autosampler Volume Delivery Linearity	12	L3		3	5 µL ^a
	12	L3	3	10 µL ^a	
	12	L3	3	20 µL ^a	
	12	L3	3	35 µL ^a	
	12	L3	3	50 µL ^a	
Cleansing Blank	13	Mobile Phase A Blank	2	10 µL	
Gradient Qualification: Accuracy/ Gradient Dwell Volume	14	GVS Spiked MP B*	GRDAB		2
	15	Mobile Phase A Blank	GRDCB		1
	16	Mobile Phase A Blank	GRDDB	1	

- a. Assumes that the Injection Volume can be modified within your injection sequence table, as for operation with the Agilent ChemStation data system. If this cannot be done for your instrument, separate methods should be written, one for each injection volume specified for the Autosampler Volume Linearity test protocol. Volume range can be changed to suit instrument requirements.



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2. Data Analysis:

The integration parameters for each of the methods specified above (see next section of the instructions) should be developed around an initial test run of a sample, to avoid the need for reprocessing of the final chromatograms. Once the PQ tests have been performed on a given instrument, the integration parameters will be stored with the method, and can typically be re-used year after year without further modifications.

An essential feature of the PQ Kit, and any instrument Performance Qualification, is that it is designed to test the operation of the entire HPLC *under your laboratory operating conditions and procedures*. You should always follow your own internal SOP's in terms of how and when reprocessing is performed, and what documentation and justification is required.

Once you feel the chromatograms are reliably integrated by your data system, you may process the results in either of two ways.

Custom Data Analysis:

First, if you prefer, you may analyze the data according to your own internal SOP's. Thus, if your laboratory already has in place an SOP calling for injector precision using 5 replicates of a sample, you would modify Sequence 01 to reflect this, and then analyze the results as per your normal SOP. When used in this manner, the contents of the PQ Kit simply represent a set of convenient, pre-made solutions.

PQ Kit Template:

The second approach is use the validated, PQ Kit Excel[™] Template, provided with the kit. Sequence PQ01 is designed to provide the data required for entry into the template. If the template is used, simply enter the peak areas into each cell of the spreadsheet. All calculations and graphing are performed automatically. The user enters the acceptance criteria (typical default values are provided in the cells, but may be overwritten).

A Demo version of the Excel template containing typical data is included on the CD-Rom. The user is encouraged to call this spreadsheet onto the screen and/or print it. The type of data required from each of the chromatograms will become obvious upon review of the typical test data provided.

The scales of each of the graphs in the template can be varied by the user to fit their data values. For example, for the autosampler precision using 10 replicate injections, simply enter the 10 peak areas and retention times for Caffeine into the area of the spreadsheet for that test protocol. The min/max, average and %Relative Standard Deviations are calculated for both areas and retention times, and the trend lines are plotted. Note that since the values of peak areas vary from one HPLC to another, the scale of the Y axis of the graph may be changed by the user so that the average lies in about the middle of the graph. Simply right click on the Y axis, and adjust the scale as desired. Consult the Excel Help menus if you are not sure how to accomplish this.

If a test is not performed, simply leave those areas of the template blank, and be sure to click on the "Not Performed" option of the drop-down boxes provided.

All test results are summarized on the front page of the Excel template. Those not performed are listed as such, and remain blank.

The front page of the Excel template is designed as a single-page sign off area for both the analyst and the reviewer, while the body of the spreadsheet contains the detailed data. After review, you may copy and paste the front sheet of the spreadsheet into your instrument logbook for documentation. Note as well that a single page results Summary Sheet has been provided in Word, WordPerfect and PDF formats. You may modify this form as desired to document the Performance Qualification to meet your laboratory requirements.

Most laboratories will find that they want to incorporate the PQ materials and procedures into their regulatory



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environment by revising their HPLC Qualification SOP's to use the PQ kit. This makes instrument qualification extremely easy, and provides the added benefit of having a single, standardized set of test procedures available laboratory and company-wide, using an available commercial product, so that instrument performance can be compared in other laboratories. This can be a very effective tool during method transfer and method troubleshooting. Also, during method development, robustness testing is facilitated by choosing instruments with very different extra-column volumes and gradient dwell volumes.

The following Table summarizes the various Test Protocol Results, and the default Acceptance Criteria assigned by the template. Remember that you are free to set your own acceptance criteria in most cases, to meet your internal SOP requirements. The following are suggested typical values only.

Summary Table of Information from Sequence PQ01:		
Preparatory Steps:		
Solution Certificate of Analysis	Enter certified concentrations of PQ Solutions into template area from the Certificate of Analysis provided.	Confirm solutions are within Expiration Date.
Instrument/Column Information	Enter relevant instrument Serial Numbers, analyst name, column serial number and any comments into template areas.	PQ Test column may be reused as long as System Suitability is met.
Instrument Hardware Tests:		
Test Protocol:	Data	Recommended Specifications
Flow Rate Qualification	Enter values obtained from flow meter, or as calculated by volume collection	± 5% of Nominal
Column Oven Temperature	Enter values found using calibrated thermocouple or thermometer	±5°C of Nominal
System Suitability:		
Rs Test Mixture	Calculate Retention Time, Efficiency, USP Tailing Factor and Resolution for Caffeine (peak #3)	Tr 4.0 - 5.0 minutes N ≥6,000 Rs prior peak ≥2.0 Rs next peak ≥2.0



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Summary Table of Information from Sequence PQ01:			
Wavelength Accuracy Qualification:			
Primary Standard Holmium Oxide (WCS) (241nm - 641nm)	For Diode Array - enter spectral maxima for up to 5 peaks as found by instrument software	Wavelength Accuracy	± 3 nm
	For VWD, enter absorbance or mV values for each λ around nominal maximum	Regression	Extrapolated accuracy @ 200 nm and 700 nm remains within specs
Secondary Standard - Caffeine (205nm and 273nm) (optional)	Use 1 st Injection of L3 solution in Precision Test- For Diode Array - enter spectral maxima for 205 nm and 273 nm bands as found by instrument software maxima	Wavelength Accuracy	± 3 nm
Sequence PQ01 Results:			
ATM Detector Noise	Use software, or manually determine baseline noise level over 7- 8 minutes for 2 nd Mobile Phase A Blank injection prior to Rs Test Mixture	Dynamic Noise @ 273 nm	Record
HPLC Extra Column Volume	Enter Efficiency for 4 peaks of Rs Test Mixture from System Suitability	EC Volume	Record
Autosampler Precision Pump Flow Drift	Enter L3 peak Retention Times, Areas from 10 injections of L3	AS Precision Tr Precision	$\leq 1.0\%$ RSD $\leq 1.0\%$ RSD
Detector Linearity (100% - 0.1%)	Enter peak areas for triplicate injections of Linearity Solutions L1-L6 Peak height entry is optional, but recommended, to allow estimate of LOD and Detector Sensitivity (normalized slope)	Area Linearity System Sensitivity Caffeine LOD	R ≥ 0.999 Examine graph for trends Record Record
Injector % Carryover	Enter found areas (if any) within the Tr of Caffeine, for Blanks immediately following L6 of the Detector Linearity set.	% Carryover	$\leq 0.1\%$ Note whether wash vial is used
Autosampler Volume Linearity	Enter peak areas for triplicate injections of L3 for each nominal injection volume	Vol Linearity	R ≥ 0.999 Examine graph for trends
Gradient Accuracy and Dwell Volume	Using Data system, or manually, measure plateau height at 100% and 50% gradient legs - enter values into template cells	Gradient Accuracy	90% - 110% Examine transitions for sharpness, drift
	For Dwell Volume, enter Tr of Uracil peak from GVS-B* blank injection at start of gradient. Determine Time of gradient onset (when gradient starts to rise off baseline of chromatogram).	Dwell Volume	Record



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3392 State Hwy 8, South New Berlin, NY 13843

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www.chemicalsolutionsinc.com
(607) 859-2706 fax (607) 859-2917

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E. Wavelength Accuracy

Two methods are available to qualify the detector wavelength; a Primary Standard using Holmium Oxide solution (241 nm - 641 nm), or a Secondary Standard Caffeine, at 205nm and 273 nm.

The Primary Standard Method is typically the better choice, since Holmium Oxide is an Internationally accepted wavelength calibration standard. Its range is limited to 241nm - 641nm, although the template software plots the wavelength accuracy over this range, and extrapolates to 200 nm and 700 nm, to show monochrometer trends.

If qualification at a lower wavelength is desired, then one of the Caffeine solutions is recommended. The template provides for the entry of Caffeine wavelength results, which, like the Holmium Oxide, may be obtained by determining the signal intensities of the flow cell at discrete wavelengths near each band of interest, or by Diode Array if that is the detector type being qualified.

Since filling the flow cell with the desired solution is common to both methods, detail of its operation will be presented in the next section.

FLOW CELL FILLING PROCEDURES:

WARNING - ALWAYS PULL THE DESIRED THROUGH THE FLOW CELL AS DESCRIBED BELOW - NEVER ATTEMPT TO PUSH THE SOLUTIONS THROUGH USING PRESSURE - SYRINGE OR TUBING RUPTURE CAN OCCUR, SPRAYING ACID INTO EYES AND CLOTHING !!!

Whichever detector type is used, the flow cell will have to be manually flushed with the desired liquids. A specially designed, spring-loaded syringe and fittings (part no. PQAFKT01), have been designed to make this job easy. The following procedures will be found to work well.

Basically, the column exit tubing (the one connecting the column outlet to the detector inlet) is used to introduce a solution into the flow cell, using a light vacuum applied to the detector outlet (waste line) with a small syringe.

- a. Fill an uncapped injection vial or small beaker, filled about ½ way with the solution you need to introduce into the flow cell.
- b. Remove the connecting tubing from the OUTLET of the HPLC column. Wipe clean, and immerse the column outlet tubing into the vial, so that the tubing inlet is beneath the liquid level in the vial.
 - The beaker or vial may have to be held or taped within the column compartment, to prevent spilling.
- c. Locate the detector Waste Line. You will need to arrange a connection between the spring loaded syringe and this outlet tubing, in order to apply vacuum.
 - On some instruments, a bulkhead fitting may be present. If so, you can disconnect the waste line, and connect the vacuum syringe tubing line, using the supplied finger-tight fitting.
 - If a plain stainless or plastic tubing is used for the waste line, use one of the short pieces of plastic tubing of appropriate diameter, to temporarily splice the syringe and detector waste tubing.
- d. Disconnect the spring loaded syringe at the luer connector. Depress the plunger, and while keeping it depressed, re-connect the syringe to the luer fitting with a twisting motion. Release the syringe barrel.
 - The barrel will retract to about the 8-9 mL mark, at which point it will be applying a small, constant vacuum to the detector outlet tubing.
- e. Due to the narrow bore tubing used for the column outlet tubing, the flow rate of the solution into the flow



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cell will be slow. Typically, it will take about a minute for the solution to flush the connecting tubing and flow cell. Note that most flow cells and connecting tubing contain a total of only 20-30 μ L of volume. Thus, once several drops have entered the syringe barrel, the flow cell will have been thoroughly flushed. You can monitor the flow rate through the tubing by following tiny bubble periodically created, which are visible moving out the plastic line into the syringe.

- f. Once the flow cell has been thoroughly filled with the desired solution - **BREAK THE VACUUM BY DISCONNECTING THE SYRINGE AT THE LEUR FITTING**. This is essential to create a quite, stable optical environment within the flow cell, without transient bubbles.

Note that the Wavelength Calibration Solution is not quantitative, but is used for wavelength accuracy only. Thus, the validity of the test is not dependent upon the exact concentration of WCS in the flow cell. However, the flow cell should always be thoroughly flushed by several cell volumes, to ensure the chemical integrity of the solution being measured in the flow cell. Flushing the cell with a volume of about 0.5 - 1.0 mL will be more than adequate for all but the largest preparative detectors.

- g. Perform the measurement of interest.

1. Primary Standard Method Using Wavelength Calibration Solution - Holmium Oxide:

- a. Uses NIST-Traceable Holmium Oxide Solution, identical in composition to SRM 2034. Refer to Appendix A for a listing of the official NIST absorbance bands spectrum.
- b. Measurement procedure for *Diode Array Detectors*
 - i. Setup Wavelength Accuracy HPLC Acquisition method with a 0 volume injection, and 0 mL/min flow rate. Some instruments permit a dummy (no injection) method. Method should acquire "All Spectra", autozeroing with purified water in the flow cell (see next item). Initially set total method run time to 10 minutes, to allow time to fill the cell with the Wavelength Solution after starting. Run time can be reduced if it is found that less time is required to complete the rest of the procedures in this section. Note that the Visible Lamp must be turned on, if available, for wavelengths over 360 nm.
 - ii. Manually fill flow cell with Purified Water, by pulling it through the cell from the column outlet tubing using the syringe provided as described previously. Once filled, disconnect the syringe.
 - iii. Start the Wavelength Accuracy HPLC Acquisition method, zeroing the detector at the beginning of the injection. Ensure that there is a short period of about 30 seconds at the beginning of the run, before starting to pull the Wavelength solution through.
 - iv. Manually fill the flow cell with Wavelength Calibration Solution, by pulling it through from the column outlet tubing using the syringe provided. Once the flow cell is thoroughly flushed, release the syringe to break the vacuum. The signal will become stable.
 - v. After the method has ended obtain the spectra of the Wavelength Calibration Solution from a stable region of the chromatogram.
- c. Measurement procedure for *Variable Wavelength Detectors*
 - i. Manually fill flow cell with Wavelength Calibration Solution, by pulling it through the cell from the column outlet tubing using the syringe provided connected to the cell outlet tubing.
 - ii. Zero the detector at 590 nm, a non-absorbing region of the Holmium Oxide spectrum.
 - iii. Select up to 5 calibration wavelengths covering the spectral region of interest.



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- iv. Document the absorbance readings in 1 nm increments several nm above and below each of the bands of interest.

2. Secondary Standard Method

- a. Uses Spectral Maxima of Caffeine, nominally at 205 nm and 273 nm, in Solution L2 or L3. Refer to Appendix A for the spectrum of Caffeine under mobile phase conditions. Solution L3 should be used if multiple injections are performed at various wavelengths, while solution L2 will produce an appropriate signal strength for most detectors if the flow cell is manually filled. Select the solution concentration that will produce a strong, mid-range signal for your detector.
- b. Measurement procedure for *Diode Array Detector*
 - i. Determine the spectral maxima of Caffeine from an injection of Solution L3 from Autosampler Precision.
- c. Measurement procedure for *Variable Wavelength Detectors*
 - i. Manual Determination
 - Manually fill flow cell with Solution L2, by pulling it through the flow cell from the column outlet tubing using the syringe provided.
 - Zero the detector at **360 nm**.
 - Document absorbance readings in 1 nm increments several nm above and below 273 nm.
 - Enter absorbance values into the spreadsheet. Wavelength of maximum signal will be graphed and determined by interpolation.
 - Repeat this procedure at wavelengths above and below the 205 nm band, if desired.



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- ii. Multiple Injection Determination (Automated - modify Seq 01 as required)
 - Set up series of methods each with different detection wavelengths, from 267 nm to 276 nm (or range chosen). Keep other conditions the same as method PQ10.
 - Inject L3 Caffeine solution for each method, obtaining peak areas or heights for each wavelength method.
 - Enter areas or heights into the spreadsheet. Wavelength of maximum signal will be graphed and determined by interpolation.

NOTE: The advantage of the multiple injection method is its full automation. However, a different method must be generated for each wavelength. Depending upon your regulatory environment, this may generate significantly more paperwork and regulatory issues.

F. Specifications

1. Typical recommended default specifications are summarized in the Summary Table (see previous section). These default values are also contained in the PQ Excel Template. The user may change these values to meet internal SOP requirements.

G. Data Analysis

1. System Suitability Analysis
 - a. The following criterion must be met for **Caffeine**, Peak #3 (Use USP Methods for measurements)
 - i. Retention time between 4-5 minutes
 - ii. Peak Efficiency not less than 6000
 - iii. Tailing Factor between 0.90 and 1.10
 - iv. Resolution of ≥ 2.0 for major peaks eluting before and after Caffeine
 - b. The system suitability chromatogram is similar to Figures 1 and 2.
2. Enter data from tests performed above into accompanying software.



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H. Typical Chromatograms:

The following figures illustrate typical chromatograms when chromatographing the samples according to the documented methods. Note the Gradient Dwell Volume determination techniques (Fig. 4).

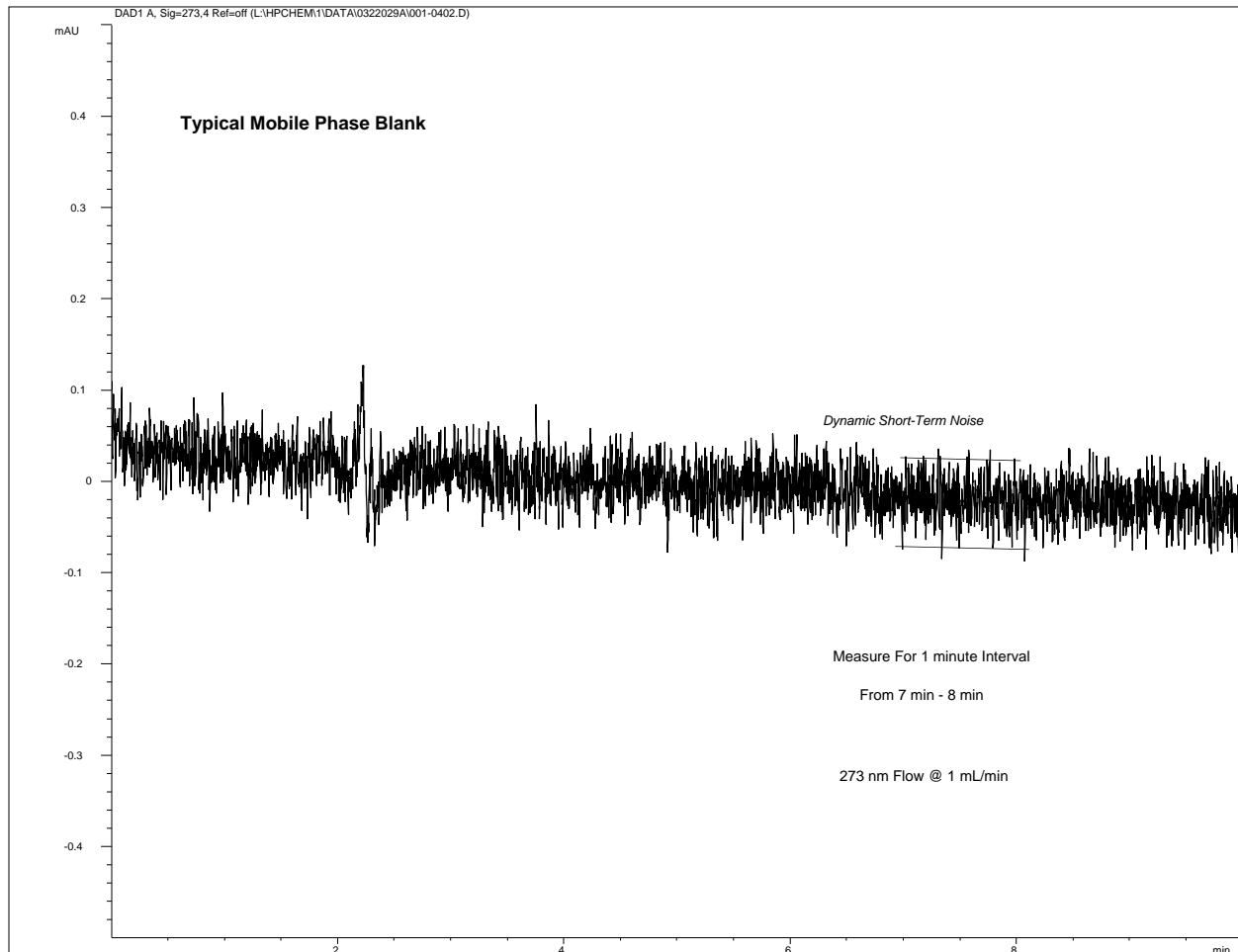


Figure 1: Mobile Phase Blank injection with method RTM1. Repeat as needed until stable, clean baseline is obtained. Determine Dynamic Short-Term noise for last clean blank with data system, or by drawing lines encompassing ca. 95% of the signal spikes, for a 1 minute interval, over the time of 7-8 minutes.



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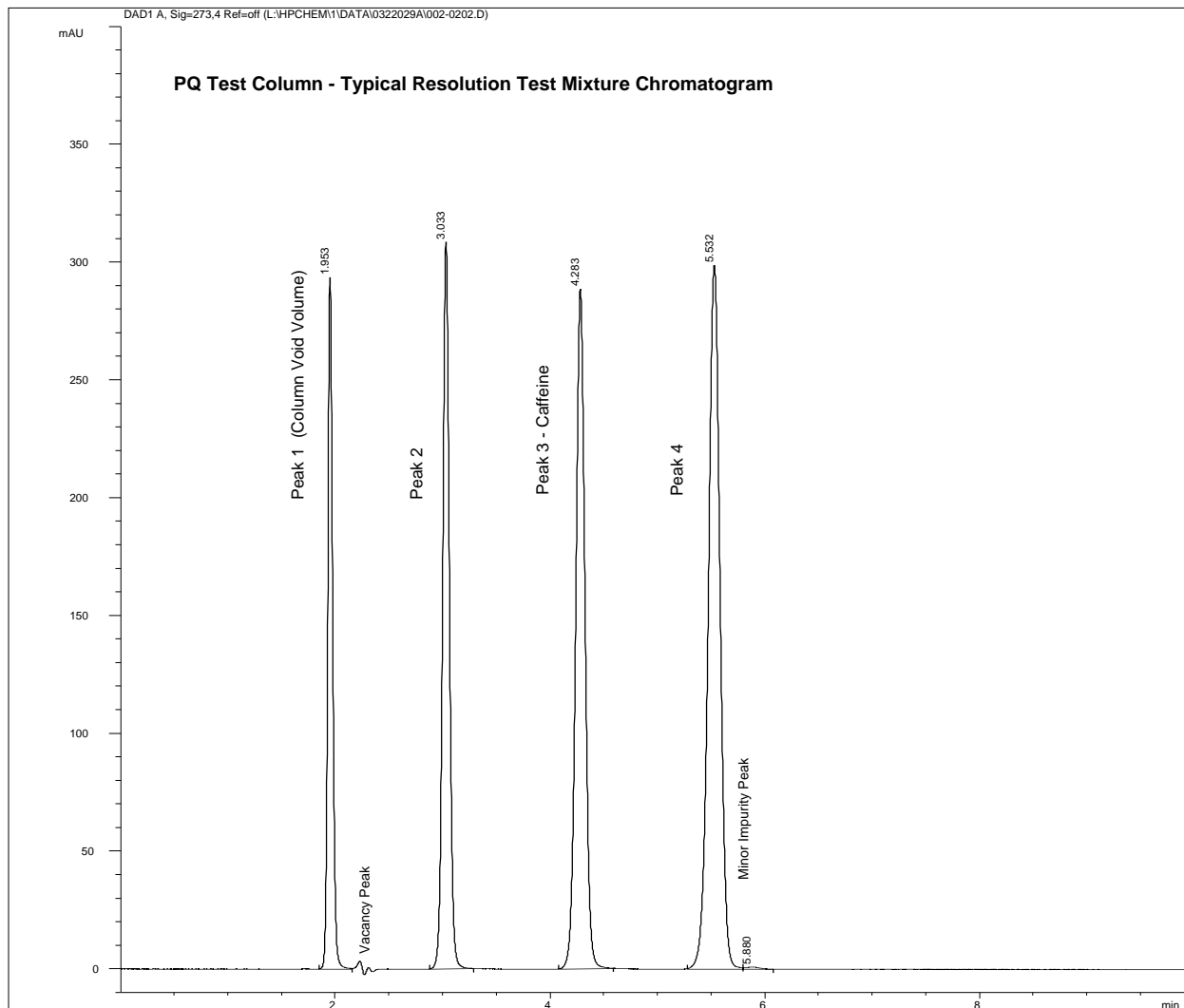


Figure 2: Resolution Test Mixture with method RTM1. Set integration parameters to avoid the Vacancy Peak present at ca. 2.3 min. There is a small isomeric impurity peak just after Peak 4. Set area reject to ignore peak.



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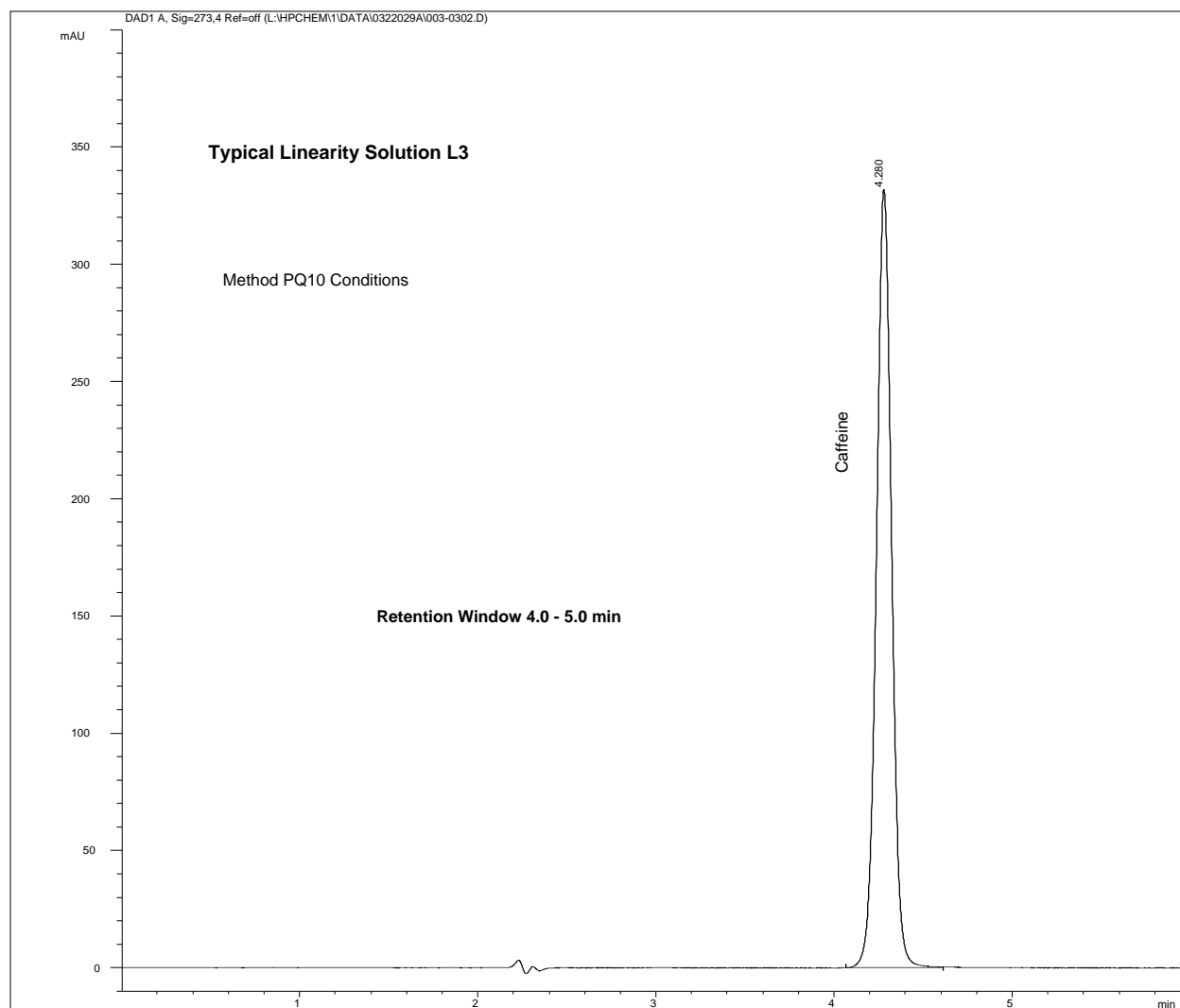


Figure 3: Typical Linearity Solution L3 sample, chromatographed according to method PQ10. Note the allowable retention time window of 4.0 - 5.0 min (see System Suitability). There are no late eluting peaks. Run time can be set to the minimum time required to reliably complete integration of the peak tail, typically 5 - 6 minutes.



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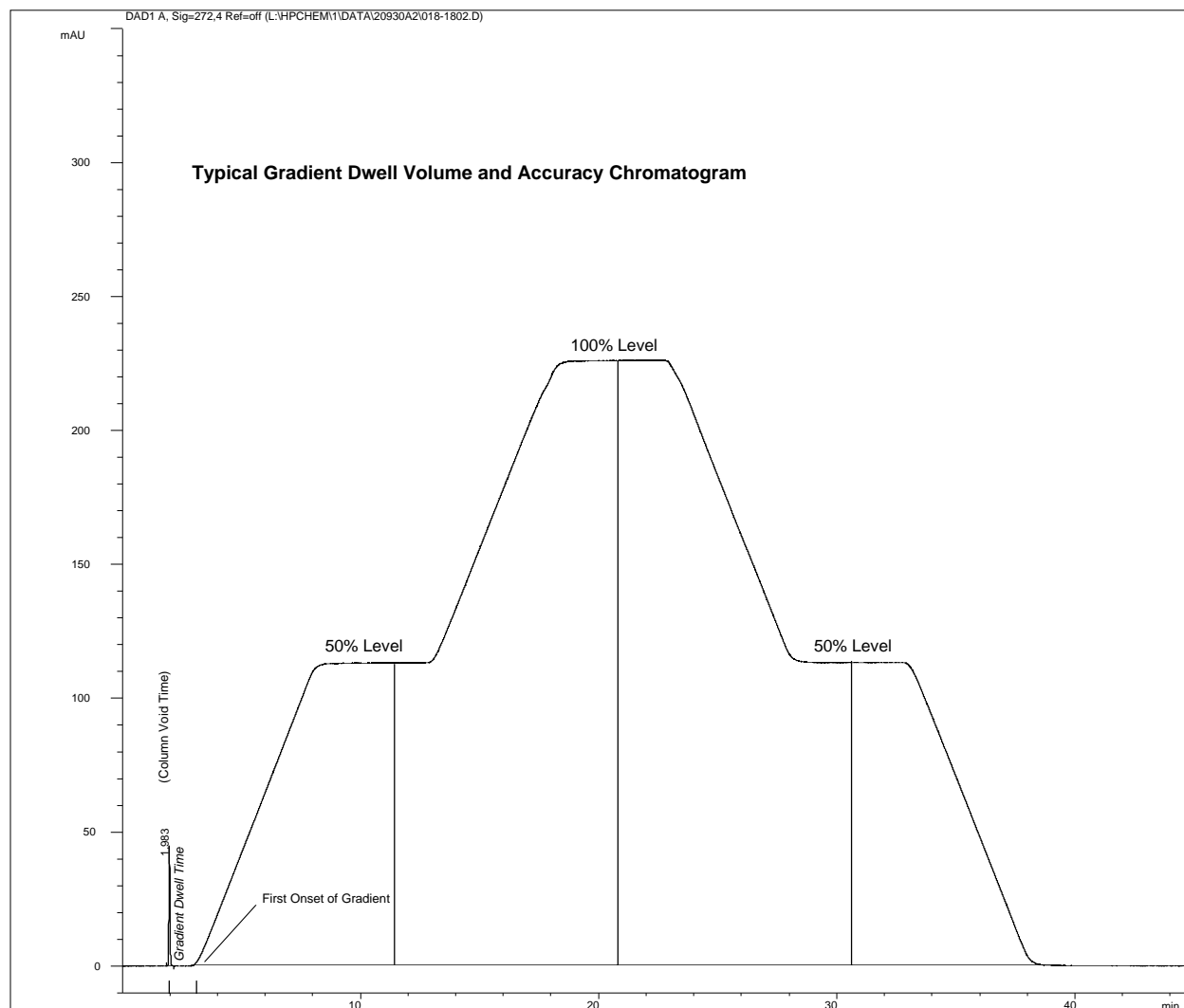


Figure 4: Typical Gradient Accuracy chromatogram with method GRDAB, for binary system (A/B*), where the B* solvent is spiked with Gradient Visualization Solution at the rate of 6 mL/L of mobile phase. A 10 uL injection of GVS-Spiked mobile phase B* is performed at the start of the method, with no gradient delay. Obtain the time of the first onset of the gradient by graphical means, using the data system or manually. Gradient accuracy is determined by comparing the relative heights of the 50% vs the 100% levels, against the expected 50%.

For C/B* and D/B* accuracy checks, inject unspiked mobile phase (use A/B* only for the Gradient Dwell Volume determination).



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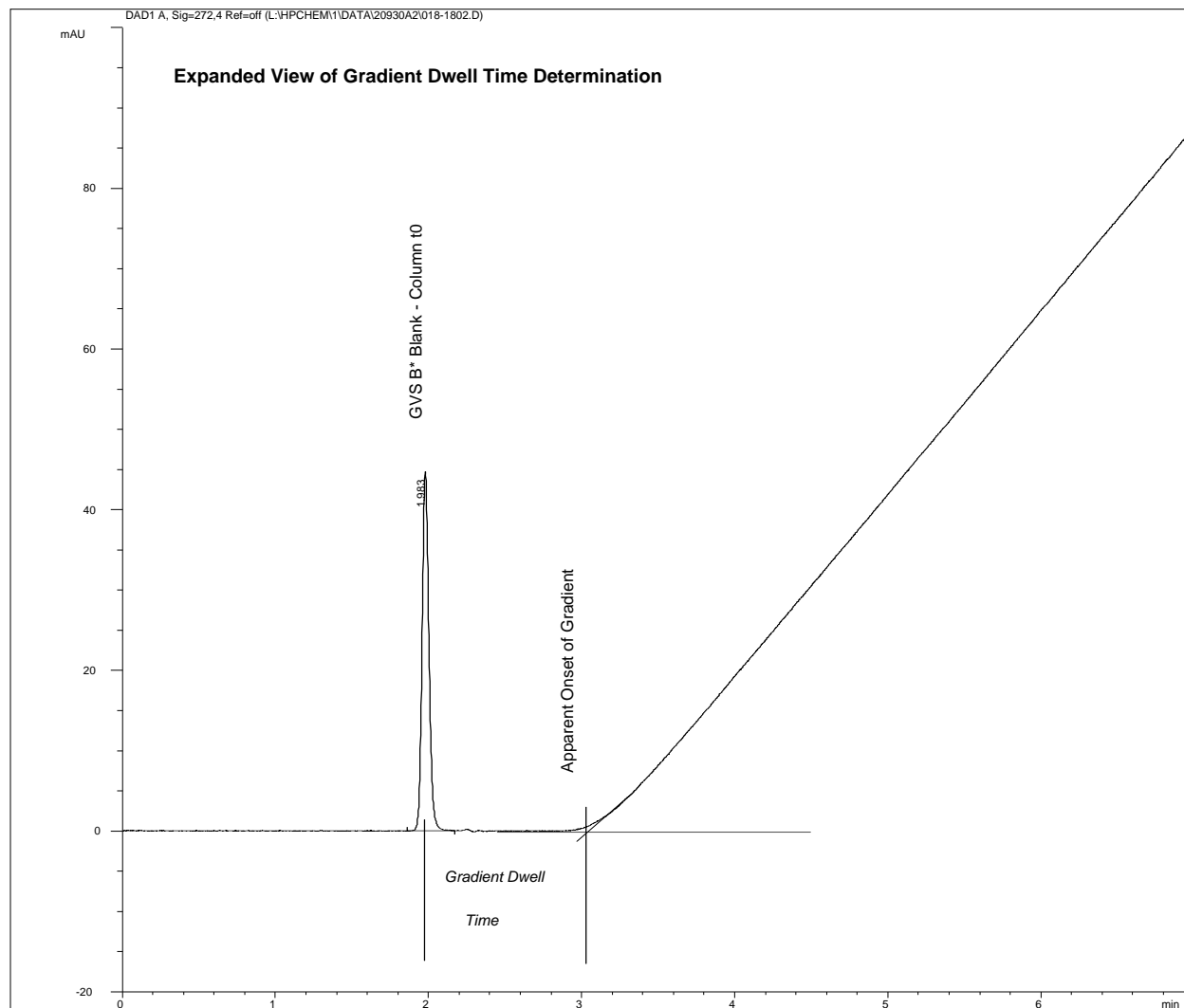


Figure 5: Expanded view of the Gradient Dwell Volume (Time) determination procedure. Gradient Dwell Volume is calculated by multiplying the Dwell Time by the calibrated flow rate.



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V. SOLUTION PREPARATION

A. **Isocratic Method Mobile Phase Preparation (13: 87[Acetonitrile:0.1%v/v Acetic Acid]):**

The following is an approximate guideline for the minimum required volumes per HPLC tested. Note that the required volumes are somewhat generous, and allow for system flushing and a safe margin remaining in the solvent reservoir.

Isocratic - all test protocols - ca. 50 injections minimum 500 mL of mobile phase/HPLC

Example Procedure for 1 L of Mobile Phase:

- Add about 500 mL of Purified Water to a 1 L graduated cylinder (TD)
- Add 1 mL of Glacial Acetic Acid (hand-pipet or serological accuracy is sufficient)
- Bring to 870 mL of volume with purified water
- Deliver to a 1L beaker

- Measure 130 mL of Acetonitrile in a suitable graduated cylinder (TD)
- Combine with the acetic acid solution in the beaker.
- Mix well. Filter/degas using a 0.4 - 0.5 μ M membrane filter. Nylon or Polycarbonate is suitable.

NOTES: The final mobile phase is 0.1% v/v in Acetic Acid. A total of 1 mL of Acetic Acid per Liter of mobile phase is used. Note that the addition of 1 mL/L of Acetic Acid results in a volume change of ca. 0.1%, which is less than the measurement accuracy of a graduated cylinder ($\pm 1\%$). Thus, an alternative procedure is simply to add glacial acetic acid to the water:acetonitrile mixture at the rate of 1 mL/L. The retention of the PQ Test components is not sensitive to the Acetic Acid concentration.

Mobile phase composition may be adjusted to meet System Suitability.

Mobile Phase is stable for 18 months when capped to prevent evaporation.

B. **Preparation of Gradient Visualization Solution (GVS)-Spiked Mobile Phase B*:**

1. Prepare Isocratic Method Mobile Phase as described in the previous section. For typical full HPLC isocratic and gradient qualification with a quaternary system, a total of about 2L of method mobile phase is recommended.
2. Measure and reserve a suitable volume of mobile phase for spiking with the Gradient Visualization Solution. A reserved volume of 500 mL will be sufficient for a quaternary gradient system.
3. Spike the reserved method mobile phase with the Gradient Visualization Solution at the rate of **6 mL/L**. Graduated cylinder accuracy of mobile phase volume, with hand-pipet or serological pipet delivery accuracy, is sufficient, since all results are internally-normalized to the 100% concentration level. Do not compensate for the volume change of the GVS solution.
4. Mix well. Do not refilter.
5. Transfer to the "B" pump/solenoid of the HPLC, and flush the fluid circuit completely, as noted above.



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C. Sample Diluent:

No sample diluent or sample preparation is required for the tests described. However, if other dilutions are desired for other experiments, all solutions (except the Wavelength Calibration Solution) may be diluted with mobile phase as required.

D. Injected Samples:

For determination of the Gradient Dwell Volume, Sequence 01 calls for the injection of the GVS-spiked mobile phase B*. To do this, simply place an aliquot of the B* spiked mobile phase in an injection vial, and inject normally.

For subsequent Gradient Accuracy Tests of the C/B* or D/B* combinations, use unspiked mobile phase as the injection blank. The Gradient Dwell Volume is measured only once, from any one of the gradient combinations, but combination A/B* is recommended as described above, for consistency.



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VI. METHODS:

A. Introduction

Due to the wide variety of HPLC instrumentation available, it is impossible to give specific pre-written methods to implement the PQ Test Protocols. However, the general conditions required for the various methods are detailed below. The user implementing these tests will normally be familiar with his or her instrumentation, and will be able to set up the instrument to acquire spectra (for Diode Array Detectors), and to decide whether wash vials should be used during testing, etc.

All isocratic test protocols for the PQ Kit are variations of a single, common base method. The base method, for convenience, is referred to as "PQ10" (PQ Test, 10 μ L injection volume).

Except for the specific Gradient Elution Tests, all other methods are similar to the base method PQ10, except for the variables being investigated. For example, if injection volume is being studied, then a series of methods based on PQ10 would be written, but with different injection volumes, such as "PQ05" (5 μ L injection), "PQ20" (20 μ L injection volume), etc.

The total number of Methods required to complete the PQ Test Protocols will depend upon the number of system components being tested. For an isocratic operational qualification, which does not perform the Injection Volume Linearity test, only 2 methods are required - the basic PQ10 method, and the RTM10 Method, which is simply the PQ10 method, but with a 10 minute, rather than 6 minute run time.

For Gradient Qualification Tests, the base method is called "GRDAB" for "Gradient, pump A/B*", where the "*" designates that the B fluid circuit is "visualized" with the addition of the Gradient Visualization Solution (GVS). If ternary or higher gradient systems are to be qualified, the same gradient method is reproduced, except that this one would test the C/B* selenoid combination, and is called "GRDCB", while a quaternary system would require a third method called "GRDDB".

Determination of Wavelength Accuracy using the Wavelength Accuracy Solution requires manually filling the flow cell with the NIST-Traceable Holmium Oxide solution. A special method is described for that purpose in the "Wavelength Qualification" section, in which the Diode Array Detector is programmed to acquire spectra without injection or flow. See that discussion for details.

The example Injection Sequence 01 refers to the methods outlined below. The user is of course, free to name the developed methods any other names. The below list is for illustration only. Methods other than those presented below may need to be written to implement the various test protocols.

Once the sequences and methods are written for a given instrument type, these should be stored and documented for re-use on other similar instruments, and for future qualifications. The user will find that once the methods and sequences have been written, future qualifications can be performed very rapidly.



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B. Isocratic Methods

1. Isocratic Base Method: **PQ10**

- a. Flow rate: 1.0 mL/min
- b. Detection Wavelength*: 273 nm
- c. Temperature: Ambient (15°C - 26°C)
- d. Injection Volume: 10 µL
- e. Run Times: about 5-6 minutes - set as required for complete integration
- f. Mobile Phase (13:87[Acetonitrile:0.1% v/v Acetic Acid])

* If the absorbance maximum of Caffeine is to be used as a Secondary Standard for Wavelength Accuracy with a Diode Array Detector, be sure to turn on spectra acquisition, according to the instructions for your detector.

2. Method **RTM10** - Resolution Test Mixture Method

- a. Same conditions as Base Method PQ10, except the following:

Run Time: 10 minutes

3. Methods PQ05, PQ20, ... PQ50

- a. Same conditions as Base Method PQ10, except for the following:

Injection Volume: 5, 20, ... 50 µL, etc.

For other method conditions not listed, use the Isocratic Base Method PQ10, and modify only those parameters required.



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C. Gradient Methods

1. Gradient Base Method: **GRDAB**

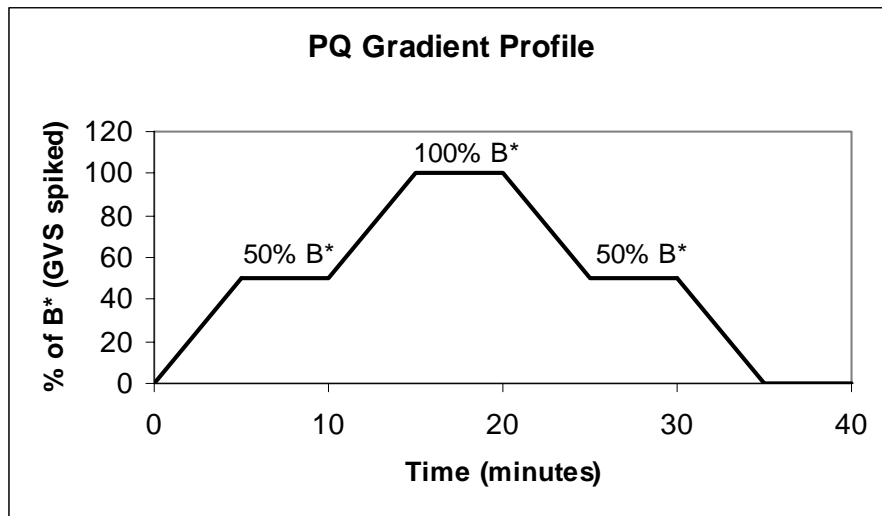
- a. Gradient qualification is accomplished by using “visualized” mobile phase in reservoir “B*”, while reservoirs A, C and D contain the regular method mobile phase. Gradient profiles are run between A vs B*, then C vs B* and D vs B* (or as many fluid circuits as are present). Method GRDAB is the base gradient method, which is modified for the other fluid combinations in the other gradient methods.
- b. All instrumental parameters (wavelength, injection volume, flow rate, temperature, etc.) are the same as base method PQ10, except for the gradient program described below.
- c. Gradient Profiles (Fluid circuit A vs B*):
 - i. Fill the “A” pump or fluid line with the (unspiked) method mobile phase (PQ10).
 - ii. Thoroughly flush the “B*” pump or fluid line with the Gradient Visualization Solution (GVS) spiked mobile phase (the * designates GVS-spiked mobile phase). Note that the B* fluid circuit must be thoroughly flushed, for good gradient accuracy results. If in doubt, perform two injections of the gradient program (see Sequence 01), ignoring the first run.
 - iii. Program your HPLC with the following solvent program, where the GVS-spiked mobile phase is contained only in the B* Reservoir. The gradient program is comprised of 5 minute segments, up to 50%, 5 minute hold, then to 100%, 5 minute hold, then down, ... etc. There is no time delay at the start of the gradient.

Method GRDAB Gradient Time/Composition Profile for Gradient Qualification of Pump A/B*		
Method Conditions: 273 nm 10 µL injection volume		Flow: 1.0 mL/min Temp: Ambient
Time:	% of Reservoir A	% of Reservoir B*
0 min	100%	0%
5 min	50%	50%
10 min	50%	50%
15 min	0%	100%
20 min	0%	100%
25 min	50%	50%
30 min	50%	50%
35 min	100%	0%
40 min	100%	0%
Allow 10 minutes re-equilibration times between methods or injections Set total run time to 50 minutes.		

A graphical representation of the gradient profile is shown in the following figure.



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2. Gradient Methods: **GRDCB** and **GRDDB**:

- a. These methods are identical to the base gradient method GRDAB, except that fluid circuits C vs B* are run. Change the gradient time table so that the composition of fluid circuit "C" replaces "A".
- b. For method GRDDB, repeat as above, but substitute fluid circuit "D" for the original fluid circuit "A".

D. Wavelength Accuracy - Primary Standard Method, for Diode Array Detectors only

1. Flow rate: 0 mL/min
2. Detection Wavelength: 241 nm
3. Slit Width: Set to the smallest Slit Width available, if selectable
4. Bandwidth: Same as used for Slit Width, if selectable
5. Spectral Acquisition: Collect all Spectra
6. Injection Volume: 0 μ L
7. Run Times: About 5 minutes

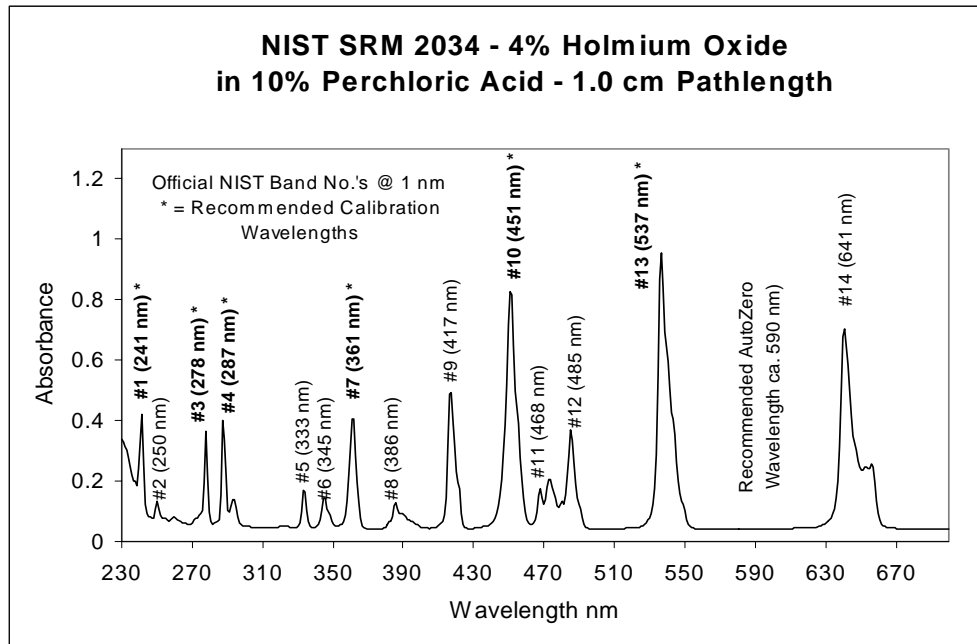
Refer to the table of official NIST absorbance bands, and the UV-Vis spectrum of NIST SRM2034 found in Appendix A.



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Appendix A:

Figure 7: UV-Vis spectrum of NIST SRM 2034 - identical to the Wavelength Calibration Solution (Item PQAHOX01). Conditions: 1.0 cm cell Ref = Water. Instrument bandwidth 1 nm.



Certified Wavelengths (nm) of Minimal Transmittance for 14 Absorbance Bands for Holmium Oxide (4% in 10% Perchloric Acid) at Six Spectral Bandwidths (Source: NIST SRM 2034, National Institutes of Standards and Technology, Gaithersburg, MD (USA), June 6, 1995).

SRM 2034 Band No.	NIST Official Bandwidth @ Spectral Bandwidth (nm)			HPLC Specification Wavelength
	1 nm	2 nm	3 nm	
1*	241.13	241.08	240.9	241
2	249.87	249.98	249.92	250
3*	278.1	278.03	278.03	278
4*	287.18	287.47	287.47	287
5	333.44	333.4	333.32	333
6	345.47	345.49	345.49	345
7*	361.31	361.16	361.04	361
8	385.66	385.86	386.01	386
9	416.28	416.62	416.84	417
10*	451.3	451.3	451.24	451
11	467.83	467.94	468.07	468
12	485.29	485.33	485.21	485
13*	536.64	536.97	537.19	537
14**	640.52	640.84	641.05	641

* Recommended wavelengths for HPLC detector qualification
** Use with some care, due to spectral shoulder.



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Figure 8: UV Spectrum of Caffeine solution in Method Mobile phase, showing absorbance maxima at 205 nm and 273 nm.

