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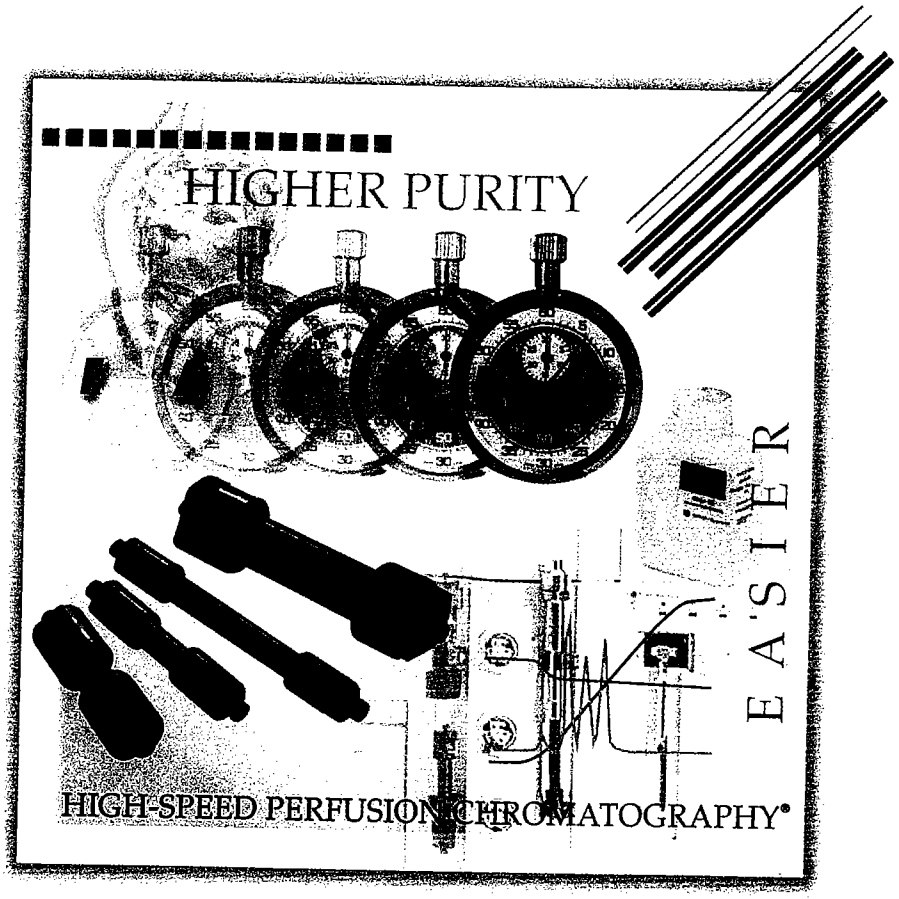
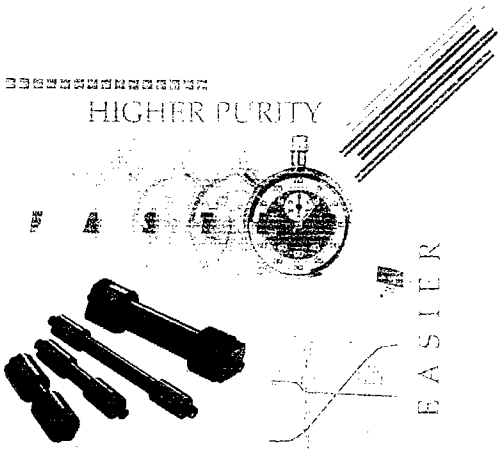
THE BUSY RESEARCHER'S GUIDE TO
BIOMOLECULE CHROMATOGRAPHY



Applied Biosystems

THE BUSY RESEARCHER'S GUIDE TO
BIOMOLECULE CHROMATOGRAPHY



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How to Use this Book

This handbook is a practical guide to developing methods for the chromatography of biomolecules using a new technique known as Perfusion Chromatography® technology. It is intended to be useful to both beginners and experienced chromatographers. It is organized into 5 major sections with a Glossary and a Reference List:

Section 1 — *Introduction* describes Perfusion Chromatography technology and how it compares with conventional chromatographic methods you may be currently using.

Section 2 — *Principles of Systematic Method Development* outlines a systematic approach to chromatography method development that is enabled by Perfusion Chromatography technology. The section includes how to define the separation problem, set up a program of experiments, evaluate the results, implement the method and troubleshoot any problems that arise. This section should be reviewed even by experienced chromatographers, since many of the ideas discussed may be different from conventional practice.

Section 3 — *Developing Your Application* covers the source materials, molecular characteristics and suggested chromatographic approaches for each major class of biomolecules (proteins, antibodies, peptides and nucleic acids).

Section 4 — *Modes of Chromatography* details the basic mechanism of each mode (ion exchange, hydrophobic interaction, reversed-phase, affinity and gel filtration) and gives a starting point method protocol, a set of minimal method development experiments, and a comprehensive list of key variables for a full method development in each mode.

Section 5 — *Basics of Chromatography* provides a concise background on the various elements of chromatographic technology for those who are getting involved in it for the first time.

Throughout, words appearing in *italics* are defined in the glossary.

The reader who requires further information on the chromatography products referred to in the book should consult the *POROS® Columns and Media Selection Guide* also available from PerSeptive Biosystems.

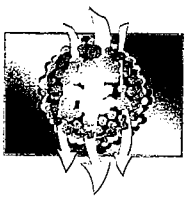
For those already familiar with chromatography, once the basic principles of Perfusion Chromatography and Systematic Method Development are understood, the handbook can be used as a practical reference for a particular application. If you are converting an existing protocol for use with POROS media, refer to the beginning of Section 3 — *Developing Your Application.*

If you are developing a new method, Section 3 — *Developing Your Application* should be used as a starting point, based on the class of biomolecule you are separating and your general approach to method development. This section should give you a good general idea of which chromatographic modes will be most useful for your application, and some specific suggestions for your kind of molecule. Section 4 — *Modes of Chromatography* will then provide the detailed method development protocols for each mode.

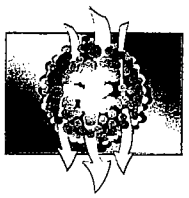
If you are new to using chromatography, after reading Section 1 — *Introduction* you should begin with Section 5 — *Basics of Chromatography,* followed by the introductory parts for each mode in Section 4 — *Modes of Chromatography.* You should then be well prepared to study Section 2 — *Principles of Systematic Method Development.* The text also has common *RULES OF THUMB* identified throughout, which should prove useful for the beginner to learn.

Users of any type of chromatographic instrumentation can benefit from systematic method development and Perfusion Chromatography media, and the experimental protocols and approaches described in this handbook have been carefully designed for use on any system. However, some specific tips are provided in Section 4 — *Modes of Chromatography* for users of the PerSeptive Biosystems BioCAD™ Workstation and BioCAD/*SPRINT*™ Systems (which were designed as workstations for systematic development).

It is our hope that, by using the techniques and products covered in this practical guide, you will discover a new power and ease of use in one of the fundamental tools of life science research. Perhaps you may even find that chromatography can be fun!



SECTION 1
INTRODUCTION



SECTION 1
INTRODUCTION

CHROMATOGRAPHY IN LIFE SCIENCE RESEARCH

Liquid chromatography is a vitally important technology in life science research. The technique provides a unique combination of capabilities. It provides the separation power needed to purify even subtle molecular variants from complex mixtures, combined with gentle chemical and physical conditions which enable recovery of biological activity for complex, biological macromolecules. Chromatography is highly scaleable and can be used for applications ranging from analysis of nanogram quantities in tiny samples on columns with sub-millimeter diameters to multi-kilogram scale production of bulk drugs in columns a meter or more in diameter.

However, for the vast majority of researchers, chromatography itself does *not* represent the focal point of their work. Instead it represents a means to an end, an important *tool* to help them isolate or identify a particular molecule of interest along the way toward answering some larger research questions (form/function etc.). Although the product of a chromatographic separation may be the most important starting point for a research project (e.g. a pure protein for X-ray crystallography studies), actually performing the chromatography itself is often viewed as the most tedious part of the project and can be frought with frustration.

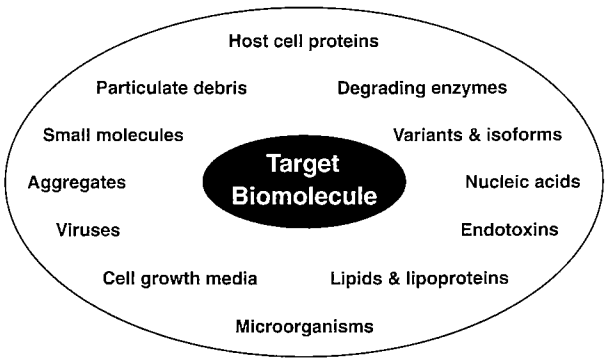


Figure 1-1. Biological systems present an unusually complex sample matrix and a difficult separations challenge.

One of the most challenging aspects for many researchers is actually developing a chromatographic separation protocol or method. The enormous flexibility of the technique, one of its most powerful features, as well as the complexity of both the target biomolecules and the biological sample matrix make it hard for the casual user to identify, much less optimize all the parameters that affect a separation. To develop a method ideally calls for a systematic experimental approach where each of the variables that could impact the separation is comprehensively tested in turn.

Unfortunately, the speed limitations of conventional chromatography media usually make it impractical to take such a systematic approach. Individual chromatographic run times on conventional media typically range from half an hour to a day or more and are the result of inherent limitations of the media (see opposite).

In an attempt to circumvent time-consuming development, researchers often rely on past experience, tips from colleagues, or previously published papers. Unfortunately, what worked in the past for one application is often not appropriate for a new research problem, leaving the researcher with a less-than-satisfactory separation and few ideas for what to do

next. When faced with a totally new separation problem, there seems to be no alternative to a lengthy, "trial and error" development process.

This handbook describes a novel systematic approach which enables effective chromatographic separations to be developed simply and quickly, allowing the researcher to spend more productive time toward their true research goals by spending less time on chromatography or by being more efficient in its use. This whole approach is made possible by the introduction of Perfusion Chromatography technology — a fundamental advance in the design and manufacture of chromatographic media developed by PerSeptive Biosystems to overcome the limitations of conventional chromatography media.

THE LIMITS OF CONVENTIONAL CHROMATOGRAPHY

It is commonly understood that with most currently available chromatography materials, one must make tradeoffs between speed, resolution and capacity. This relationship is often depicted as a triangle with each apex labelled with one of these parameters. The practical ramification is that there is an interdependence among these parameters, and that an increase in resolution or capacity must be at the expense of speed, or an increase in speed must be at the expense of resolution or capacity.

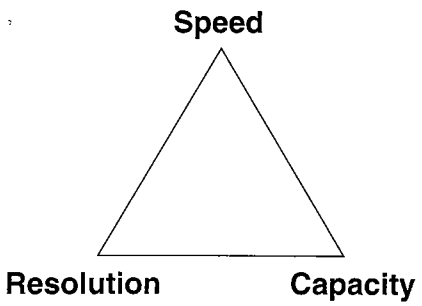


Figure 1-2. The tradeoff with conventional media between speed, resolution and capacity is depicted by many media manufacturers as a triangle.

The reason for this tradeoff lies in the nature of the particles that make up conventional chromatography media. In order for a chromatographic separation to occur, solute molecules must interact with the surface of the media particles. A typical chromatography particle is highly porous (with pores in the 100-1000 Å range) in order to maximize the internal surface area for binding. Solute molecules are carried to the perimeter of the particles by the liquid stream as it flows through the packed bed of the column. Transport of the solute molecules to the *inside surfaces of the particles occurs by diffusion within the pores (intraparticle diffusion).*

Diffusion is a slow process, especially for large macromolecules, and becomes the limiting factor in a conventional chromatography separation. As flow rate across the column increases, there is less time available for this intraparticle diffusion to occur, therefore less ability for solute molecules to interact with the surface area inside the particles. Band-spreading increases, and resolution and capacity are lost if the flow rate is too high. For first-generation biological chromatography packings (so-called soft gels) with 90-200 µm particle diameters, the typical time for a single separation run is on the order of hours or even days.

The introduction of modern *high performance liquid chromatography (HPLC)* materials brought a 10-fold advance in speed over the first-generation packings. HPLC enables higher speed separations by a reduction in the particle diameter to the range 3-30 µm. This reduces the distance for solute diffusion within the particles, and thus allows operation at higher flow rates. However, the relative speed improvement with HPLC is not the result of any fundamental advance in the pore structure of the particles themselves. Diffusion is still limiting in conventional HPLC columns. A typical "lab scale" size HPLC column usually cannot be run much faster than 1 ml/min (30 minute to 1 hour total run time) without an unacceptable loss of resolution and capacity.

PERFUSION CHROMATOGRAPHY — A NEW APPROACH

Perfusion Chromatography technology was introduced by PerSeptive Biosystems in 1989 and represents a fundamental advance in the design of chromatography particles. POROS Perfusion Chromatography media are designed to radically speed access to the interior of the chromatography particles by overcoming the diffusional mass transfer limitations of conventional chromatographic media.

Unlike conventional chromatography particles, POROS particles have two distinct types of pores — large *throughpores* that transect the particle and short *diffusive pores* that branch off from the throughpores, providing a large internal surface area for solute/particle interactions to occur. Flow through the packed column produces a pressure differential across each particle that induces flow through the throughpores. Sample molecules are carried by this throughpore flow into the interior of the particle and into contact with the network of diffusive pores. Since the length of the diffusive pores is small in comparison to the total particle diameter (typically less than 1 μm), the time required for sample molecules to diffuse to and from internal binding sites is very short.

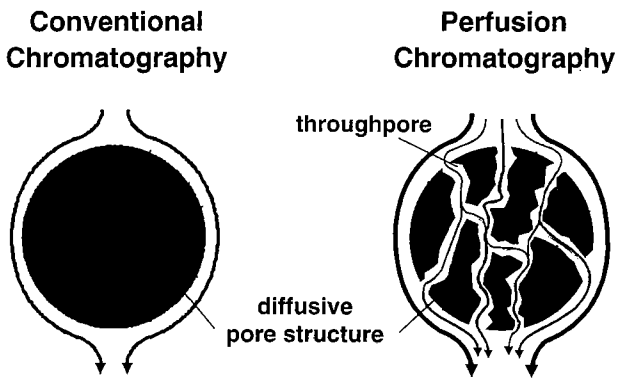


Figure 1-3. Pore structure and mass transport in conventional and perfusive particles

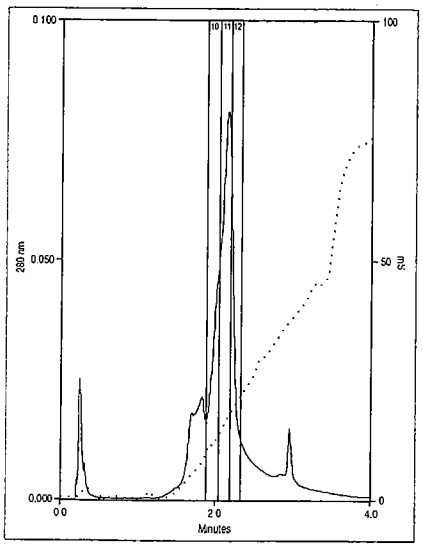
The combination of intra-particle flow and short diffusive pores effectively serves to access the entire surface area inside the particles much more rapidly than would be possible with conventional media, which rely solely on diffusion to achieve the same effect. Separations may therefore be carried out much faster on Perfusion Chromatography media with little or no loss in resolution or capacity. A more complete theoretical treatment of Perfusion Chromatography can be found in the companion booklet, *An Introduction to Perfusion Chromatography,* also available from PerSeptive Biosystems.

APPLICATIONS OF PERFUSION CHROMATOGRAPHY

The speed advantage of Perfusion Chromatography is on the order of 10 times over that of conventional HPLC columns, bringing typical separation times for a lab scale size column down to 3 - 5 minutes. In some ways, Perfusion Chromatography with POROS media redefines the way that researchers use and view chromatography in life science research. Many of the old trade-offs no longer come into play, and new application possibilities are opened.

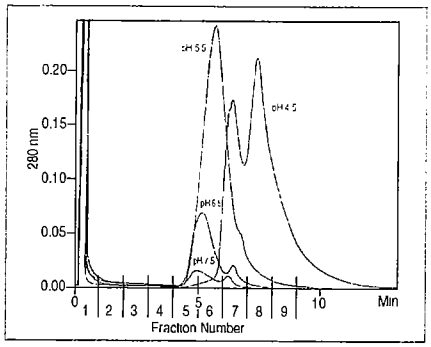
Improve and Simplify Method Development

Perhaps the most important advancement enabled by Perfusion Chromatography is a practical, systematic approach to the development of a separation method, which overcomes many of the problems discussed above. The short run times make it possible to complete a whole series of experiments in the same time it would take to make just a single run on conventional chromatography media. With this capability, the researcher can examine the critical separation variables *one at a time,* building a comprehensive picture of the behavior of the system, which makes it straightforward to design and test an optimal protocol. This systematic method development approach is the primary subject of this handbook and can be used on any chromatography system, although



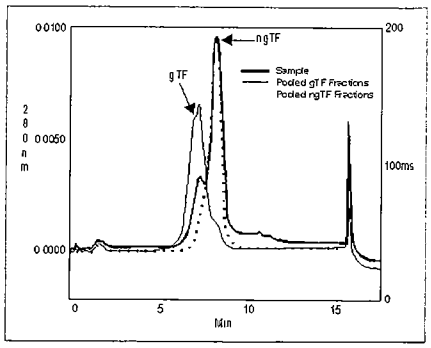
Sample:	Inclusion bodies solubilized in 10 M urea, 50 mM Tris, 5 mM EDTA, pH 8.0. Filtered and diluted 1:1 prior to injection (500 µl)
Column:	POROS HQ/M 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM Tris/bis-tris propane, pH 9.0
Eluent (B):	20 mM Tris/bis-tris propane, pH 9.0 + 3M NaCl
Flow Rate::	10 ml/min (3600 cm/hr)
System:	BioCAD/*SPRINT* system
Detection:	280 nm
Elution:	0 - 25% B in 15 CV

Lab scale purifications are routinely performed in 3-5 minutes, as in the first step of *Giardia lamblia* recombinant vacuolar ATPase A subunit purification from *E. coli* inclusion bodies. SDS PAGE revealed fractions 11 and 12 contain protein of interest with only two minor contaminants still remaining. From work conducted by Elena Hilario and Dr. Peter Gogarten at Univ. of Connecticut.



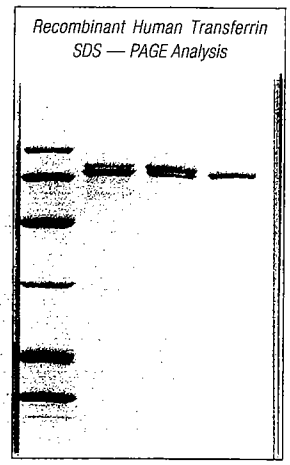
Sample:	50 µl *E. coli* cell culture supernatant (2.5 mg total protein)
Column:	POROS HS/M 4.6 mmD/100 mmL
Starting Buffer (A):	33 mM HEPES/MES/acetate, pH's as shown
Eluent (B):	33 mM HEPES/MES/acetate + 1.5 M NaCl
Flow Rate:	5 ml/min (1800 cm/hr)
System:	BioCAD Workstation
Detection:	280 nm
Gradient:	0 - 100% B in 15 CV

pH is one of the variables that can be systematically and conveniently explored with the short run times of Perfusion Chromatography, as shown in these overlaid traces of recombinant human heat shock protein (hsp 60) run on a POROS HS cation exchange column. pH 6.5 yielded fractions containing the least amount of contaminating proteins, and the highest concentration of the target molecule. After a final immunoaffinity purification to ensure no contamination with its bacterial counterpart, the hsp 60 was greater than 95% pure. PerSeptive Biosystems Application Note PA 419.

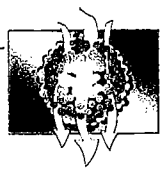


Sample:	BHK cell culture supernatant, 250 µl
Column:	POROS QE/M 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM Tris/bis/propane, pH 8.5
Eluent (B):	20 mM Tris/bis/propane, pH 8.5 + 1 M NaCl
Flow Rate::	5 ml/min (1800 cm/hrr)
System:	BioCAD/*SPRINT* system
Detection:	280 nm
Elution:	0-25% B in 45 CV

The result of taking a systematic approach to methods development. In this example, attempts with conventional chromatography media to develop an effective purification of recombinant non-glycosylated human transferrin (nghTF) from natural glycosylated form (gTF) present in tissue culture medium were met with frustration for two years. Using Perfusion Chromatography, this method was developed in a single day. Lanes 2-4 of electrophoresis gel show increasing purity of nghTF (lower band) through purification process. From work conducted by Dr. Anne B. Mason, U. Vermont College of Medicine. PerSeptive Biosystems Application Note PA 413.



Lane 1: Molecular weight markers
Lane 2-4: Progression through purification process showing increased purity of nonglycosylated hTF (lower band)



PerSeptive Biosystems has developed its own line of instrumentation (BioCAD family of systems) that further facilitates the process.

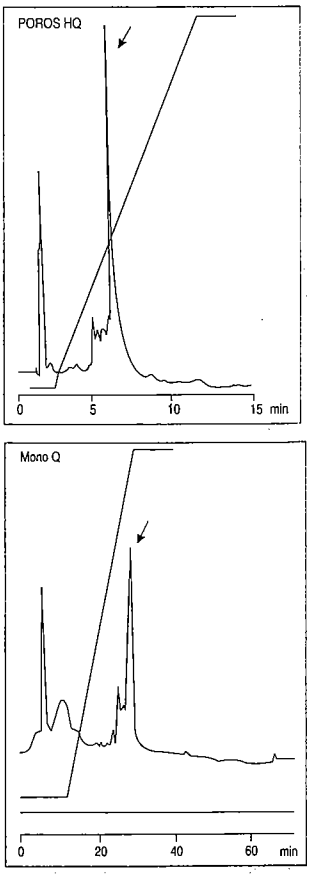
Taking the systematic Perfusion Chromatography approach can mean the difference between quickly zeroing in on the separation conditions that yield the molecule of interest in highly pure form, or experiencing frustration over yet another setback.

Even in cases where time and sample are limited, the high throughput of Perfusion Chromatography columns can make effective method development possible with fewer experiments than conventional techniques. For example, relatively shallow gradients can be used in ion exchange scouting runs, eliminating the need for gradient slope optimization once proper pH conditions are found. (See Section 4 — *Modes of Chromatography* for more details.)

Methods developed for use in an industrial quality control or production environment will need to be validated to confirm and document that the results meet the intended objectives. The systematic development approach is a good foundation for later validation, since the comprehensive information developed is essential to determining operating limits. The speed of Perfusion Chromatography allows final validation experiments to be performed quickly and efficiently.

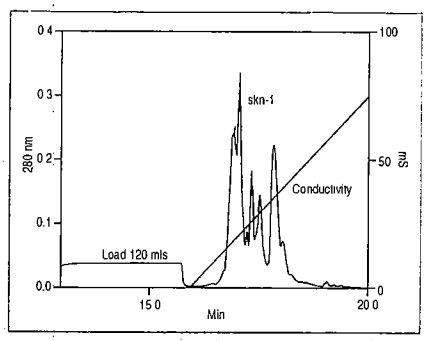
Enhance Recovery of Biological Activity

Compared to conventional chromatography, the fast run times of Perfusion Chromatography mean that the target molecules spend less time out of their natural state while being purified, under conditions that may promote denaturation. This also means less time in contact with potentially degrading enzymes that may be present in the sample. All of these factors can have a significant impact on the yield of biologically active product.



Sample:	2.5 ml partially purified and dialyzed into start buffer
Column 1:	POROS HQ/F 4.6 mmD/50 mmL
Column 2:	MonoQ® HR 5/5
Starting Buffer (A):	20 mM Tris HCl, 1 mM DTT, pH 7.5
Eluent (B):	Buffer A + 1 M KCl
Flow Rate 1:	4 ml/min
Flow Rate 2:	1 ml/min
System:	FPLC® System
Detection:	280 nm
Elution:	5 - 100% B in 40 CV

The purification of recombinant *E. coli* Mrr restriction enzyme on POROS anion exchange column was completed almost 5 times faster than the previously developed method on the conventional column. The enzyme was recovered from the POROS column in higher quantity and retained higher biological activity. From work conducted by Dr. J. Benner, New England Biolabs.



Sample:	120 ml pooled fractions from initial crude purification (0.5 - 1 mg/ml total protein)
Column:	POROS S/M 10 mmD/100 mmL
Starting Buffer (A):	20 mM HEPES, pH 6.5
Eluent (B):	20 mM HEPES, pH 6.5 + 1.5 M NaCl
Flow Rate: 1:	Sample load: 7.5 ml/min (575 cm/hr)
Flow Rate: 2:	Elution: 20 ml/min (1525 cm/hr)
System:	BioCAD/*SPRINT* system
Detection:	280 nm
Elution:	0 - 100% B in 15 CV

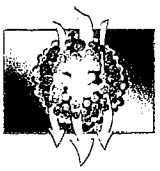
In the final step of the purification of recombinant skn-1 DNA binding protein, 120 ml of sample was processed in less than 20 minutes, eliminating the need for a separate concentration step that might have led to product loss. The yield was 25 mg at greater than 99% purity, sufficient for subsequent NMR studies. From work conducted by Dr. Gerhard Wagner and Dr. Dara Gilbert, Harvard Medical School. PerSeptive Biosystems Application Brief PA 421.

Simplify or Eliminate Sample Preparation Steps

The recovery of product (activity *and* mass) can be affected by sample preparation steps that take place before, between, or after the chromatography steps in a purification protocol. Concentration by ultrafiltration is often used at the outset of a purification to reduce the initial volume of a dilute sample, often simply to reduce the time required to load the chromatography column at the low flow rates necessitated by conventional media. In between purification steps, fractions coming off one chromatography column frequently need to be exchanged (often by dialysis) into a new buffer for the next column. When the first chromatography column is relatively large, fraction volumes for dialysis can also be large.

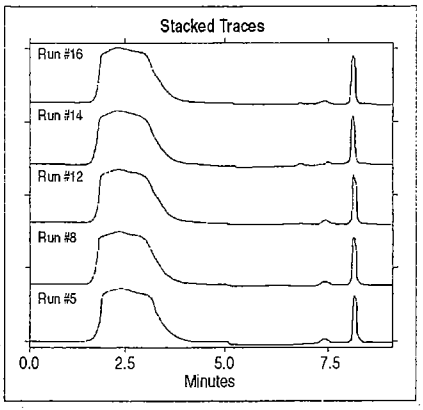
In both cases, the high capture efficiency at high flow rates of Perfusion Chromatography media can be employed to simplify or eliminate these steps, as well as the additional sample manipulation steps that can lead to product loss. In the first case, the large sample volume can be applied directly onto the chromatography column since the time to do so will no longer be a rate-limiting step. When the buffer conditions must be changed, it may be faster and simpler to *dilute* the original fraction in order to establish conditions for binding to a second column, after which you can use a high flow rate to apply and concentrate the sample on the column.

A second way in which POROS media can be used to simplify sample preparation stems from their pH stability. In the case of reversed-phase purification of synthetic oligonucleotides, detritylation with 2% TFA (which is normally done in solution) can be performed on-column in minutes, saving considerable time and possibly product loss through extra handling. This also holds true for Oligo R3™ medium, a PerSeptive Biosystems' reversed-phase packing specifically designed for preparative purification of synthetic oligonucleotides. On-column detritylation should not be performed with conventional silica C18 packings because of their limited ability to withstand the low pH of the detritylation conditions.



Reduce Column Size

In applications where large amounts of sample need to be purified, high speed Perfusion Chromatography enables the use of small columns. Since a perfusive column can be operated many times faster than a conventional column, several runs can be made on a smaller column to purify as much product in the same time as a scaled-up conventional column. This approach reduces the cost and uncertainty in scaling up, especially in the research environment.



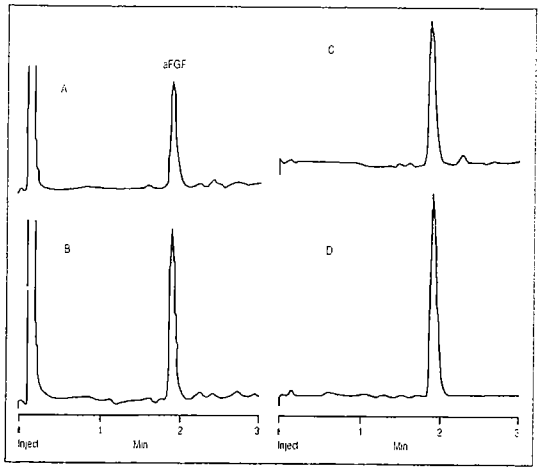
Sample:	Each 250 µl crude goat antiserum
Column:	Human transferrin immobilized on POROS EP/M 4.6 mmD/100 mmL
Starting Buffer (A):	PBS, pH 7.2
Eluent (B):	5 mM HCl
Flowrate:	2 ml/min (725 cm/hr)
System:	BioCAD workstation
Detection:	280 nm
Elution:	Step to 100% B

Use of POROS activated affinity to selectively purify antibody of desired specificity (anti-transferrin) from polyclonal population. Five of 16 separate injections are shown demonstrating how repeat cycling can be conveniently used to process larger samples. PerSeptive Biosystems Application Note PA 412.

Eliminate Analysis as a Bottleneck

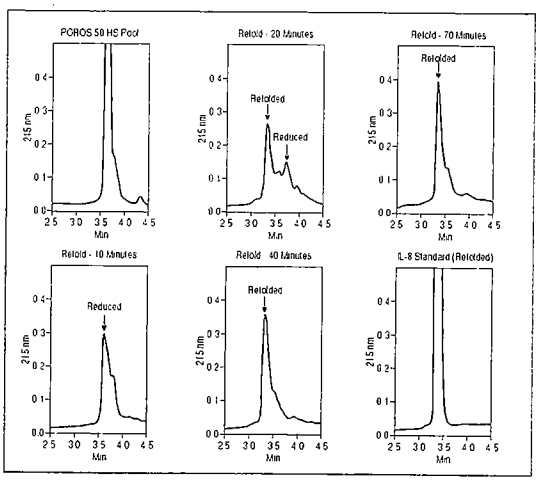
In addition to purification, chromatography is also often used for analysis (e.g. to analyze fractions of a purification step, to detect the presence of the target molecule in biological samples, etc.). When there are a large number of fractions or samples to assay, the analysis portion of the research project can represent a significant bottleneck. If a chromatographic assay can be adapted to POROS, the productivity of the lab can be increased significantly.

Not only can the impact be felt from a strict time-saving point of view, but in some cases from an efficiency point of view as well. For example, if the assay can be reduced to a few seconds or minutes, the output of a purification column can be monitored for the product in real time, and fractions only collected when product is present. This approach [which is embodied in the PerSeptive Biosystems' Real-Time Process Monitor (RPM®) System] can significantly reduce the number of fractions which must be collected and analyzed, and actually increase the yield of pure product.



Sample A:	Prepared cell paste suspension
Sample B:	In-process cell lysate
Sample C:	Anion exchange capture column product
Sample D:	Final affinity purification column product
Column:	POROS R1/H 4.6 mmD/50 mmL
Starting Buffer (A):	0.1% TFA in water
Eluent (B):	0.1% TFA, 80:20 acetonitrile-water
Flow Rate::	4 ml/min (1450 cm/hr)
System:	Conventional HPLC
Detection:	280 nm
Elution:	10 - 35% B in 20 s
	35 - 51% B in 2:25 min

A reversed-phase chromatographic assay was adapted to POROS to facilitate the quantitation of recombinant acidic Fibroblast Growth Factor (aFGF) during all stages of fermentation and purification. Three minute run times meant that *E. coli* expression levels could be closely monitored, and a full process recovery analysis (60 to 80 samples) could be completed in 4 hours. All samples 20 µl. Reprinted from *J. Chromatogr. A,* 663, DePhillips *et. al.,* Reversed-phase high performance liquid chromatography assay for recombinant acidic fibroblast growth factor in *E. coli* cell suspensions and lysate samples, 43-51, 1994 with kind permission of Elsevier Science, The Netherlands.



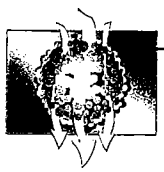
Sample:	50 µl aliquots from refolding reaction
Column:	POROS R2/M 4.6 mmD/100 mmL
Starting Buffer (A):	0.1% TFA in water
Eluent (B):	0.1% TFA in 95% acetonitrile
Flow Rate::	8.5 ml/min (3000 cm/hr)
System:	BioCAD workstation
Detection:	215 nm
Elution:	1 - 75% acetonitrile

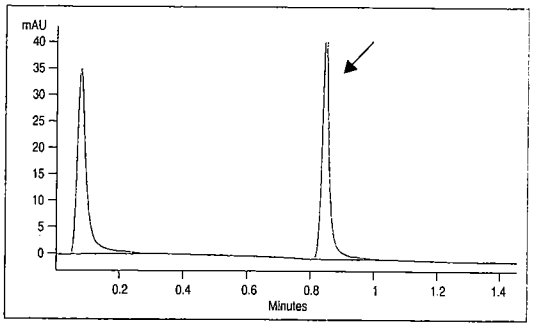
The first step in the purification of recombinant Interleukin-8 (IL-8) required solubilization of the cytokine from *E. coli* inclusion bodies with 8 M urea. The denatured product recovered from cation exchange column had to be refolded before loading onto the second (hydrophobic interaction) column. The existing method called for an overnight reaction to ensure refolding was complete. A rapid POROS reversed-phase separation was developed that allowed the refolding reaction to be monitored in "real time". It revealed that refolding was >90% complete within 70 minutes, as indicated by peak retention time shift in the series of chromatograms shown. The extra waiting time built into the original procedure was eliminated, creating time efficiencies in addition to those already realized in converting the purification method itself to POROS. PerSeptive Biosystems Application Note PA 422.

Develop Novel Assay Techniques

The high speed of Perfusion Chromatography enables a number of highly novel assay techniques. For example, it is possible to immobilize antibodies on POROS media and carry out on-column immunoassays using ImmunoDetection™ cartridges. The very short diffusion paths, high surface area, and rapid washing can reduce the many hours required for conventional microplate-based immunoassays to a few minutes, and allow complete automation with conventional HPLC instrumentation. (See the booklet *Introduction to ImmunoDetection,* available from PerSeptive Biosystems, for more details on this technique.) Enzymes immobilized on POROS perfusive supports (available as Poroszyme™ products) allow protein digestion and other analytical processes to proceed far more quickly and efficiently, without enzyme autodigestion.

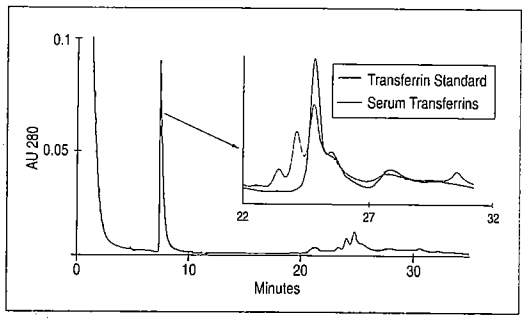
The short run times of perfusive media also make complex, multi-column and "hyphenated" analyses much more practical. For example, an ImmunoDetection cartridge immobilized with antibody can be used to quickly separate a particular type of biomolecule from a complex biological sample, and the bound target can then be eluted onto a reversed-phase column to separate the various isoforms. Perfusion Chromatography media packed into capillary columns allow for more efficient utilization and detection of sample when coupled directly to mass spectrometry instrumentation. The combination of high speed media and flexible, automated instrumentation (such as the PerSeptive Biosystems' INTEGRAL™ Micro-Analytical Workstation) enable the development of multi-dimensional chromatographic assays to solve many challenging analytical problems that could not be addressed before.





Sample:	25 µl reference standard (approx. 5 µg protein)
Column:	POROS 20 EP ID cartridge (2.1 mmD/30 mmL) with immobilized Lewis Y antigen
Starting Buffer (A):	PBS
Eluent (B):	0.1 phosphoric acid, pH 2.3 + 0.15 M NaCl
Flow Rate::	2 ml/min (3465 cm/hr)
System:	HP 1090
Detection:	280 nm
Elution:	Step to 100% B

Adaptation of an immunoassay to a POROS column. Lewis Y antigen is covalently attached to an activated affinity cartridge and specifically binds the target analyte, a chimeric IgG, which is eluted with acid and quantitated by OD 280 nm. The assay takes less than 2 minutes and is highly reproducible (<5% CV), eliminating limitations of the conventional assay techniques and lending itself to automation with readily-available HPLC equipment. Schenerman and Collins, *Anal. Biochem.* 217: 241-247. Reprinted with permission.



Sample:	1/10 human serum (50 µl)
Column 1:	POROS ID cartridge immobilized with anti-human transferrin antibody
Column 2:	POROS HQ/H 2.1 mmD/100 mmL
Starting Buffer (A):	50 mM Na Borate, pH 9
Eluent (B):	Buffer A + 1 M NaCl
Flow Rate:	2 mL/min
System:	INTEGRAL workstation
Detection:	280 nm
Elution:	6.5 - 11.5% B in 30 CV then to 50% B in 10 CV

Coupling of POROS immunoaffinity and anion exchange columns for simultaneous determination of target molecule concentration and isoform analysis. In this example, transferrin is selectively purified from the serum sample by the first immunoaffinity column. Concentration can be determined by peak area. After detection, the eluent from the ID cartridge is passed directly onto a POROS HQ column. The elution profile, when compared to that of transferrin standard (enlarged area, colored trace), indicates the presence of two additional isoforms in the serum sample.

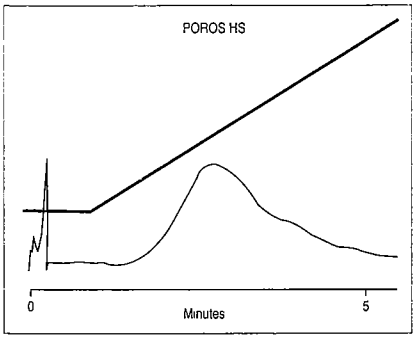
PERFUSION CHROMATOGRAPHY IN A RANGE OF TECHNIQUES

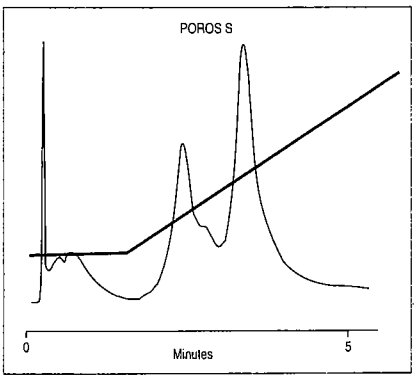
The fundamental performance characteristics (resolution and capacity at high speed) of Perfusion Chromatography technology are directly related to the unique pore structure of the poly(styrene-divinylbenzene) (PSDVB) particles that form the basis for all POROS media. The base particles themselves, being hydrophobic in nature, are available for *reversed-phase* applications.

To create Perfusion Chromatography media suitable for other modes, the base PSDVB particles are subsequently coated with a hydrophilic polymer and then functionalized for *ion exhange, hydrophobic interaction, affinity, activated affinity,* and *metal chelate* chromatography. The coating process does not interfere with the unique mass transport properties of the POROS particles, thus ensuring that the speed benefits of Perfusion Chromatography are available in each case.

POROS CHEMISTRIES

Ion Exchange	Reversed-Phase
HQ strong anion	R1
QE strong anion	R2
PI weak anion	
DEAE weak anion	**Affinity**
	A protein A
HS strong cation	G protein G
SP strong cation	HE heparin
S strong cation	MC metal chelate
CM weak cation	
	Activated Affinity
Hydrophobic Interaction	EP epoxide
HP2 high density phenyl	AL aldehyde
PE phenyl ether	HY hydrazide
ET ether	NH amine
	OH hydroxyl





Sample:	20 µl crude peptide (10 mg/ml) in start buffer
Column 1:	POROS HS/M 4.6 mmD/100 mmL
Column 2:	POROS S/M 4.6 mmD/100 mmL
Starting Buffer (A):	20 mM Trls/HCl, pH 8.5
Eluent (B):	20 mM Trls/HCl, pH 8.5 + 0.5 M NaCl
Flow Rate:	5 ml/min (1800 cm/hrr)
System:	HP 1050
Detection:	214 nm
Elution:	0 - 40% B in 5 min

Separation of a 28 residue synthetic vasoactive peptide (VIP) on different POROS cation exchange chemistries. A less hydrophobic functional group coupled with a lower ligand density on POROS S provided much better selectivity for this very basic, very hydrophobic peptide. From work conducted for LSU medical school.

Within certain chromatographic modes, there is a further range of *selectivities* available in POROS media. For example, there are four different POROS media available for cation exchange chromatography, each with a different functional group and/or ligand density. Depending on the nature of the sample, the separation profile on each packing in a series can be markedly different — a factor that can be exploited when trying to develop an effective separation method.

The range of selectivities on conventional high performance chromatography media is typically not as extensive as that found on POROS. Whether this relates to a manufacturing issue or not, even if a broader range of chemistries were available, the speed limitations of conventional media would make it impractical to thoroughly test them. This is not the case with Perfusion Chromatography media where the speed of the runs makes it convenient to bring all the power of differing selectivity to bear on a separation problem as required.

To reduce the cost associated with trying several different column chemistries in order to take advantage of selectivity, POROS media are available in a unique Self Pack® format (in addition to the prepacked column format).

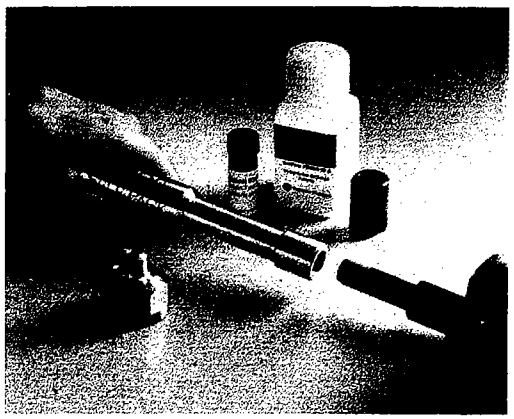
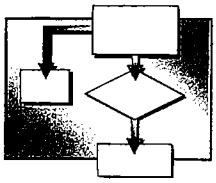


Figure 1-4. The Self Pack system gives researchers the ability to pack their high performance POROS columns, using their existing chromatography system.

One common technique for which there is not an analagous POROS product is *gel filtration* (or *size exclusion*) chromatography. Gel filtration relies on diffusion within the particles to create the "sieving" effect that fractionates based on molecular size. The effective pore structure of Perfusion Chromatography particles minimizes this diffusion so POROS media is not well suited for gel filtration. However, this does not mean that POROS cannot be used to advantage for a separation that currently uses gel filtration. In some instances, a Perfusion Chromatography alternative can be found. If possible this option should be explored, especially if the acute speed and capacity limitations of gel filtration pose a problem.

Refer to the companion piece, *POROS Columns and Media Selection Guide*, also available from PerSeptive Biosystems for a complete description of the POROS chemistries, column geometries and product formats available.



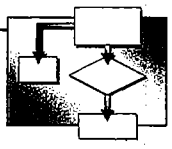
SECTION 2
PRINCIPLES OF SYSTEMATIC METHOD DEVELOPMENT

DEFINE

The Target Molecule & Sample
Class of Molecule
Molecular Characteristics
Sample Source
Analytical Methods
Screening Analysis vs. Final Analysis
Key Analytical Techniques
Tracking of Peaks
Separation Goals
Analytical
Preparative
Overall Separation Strategy
Resources
Sample
Time
Equipment

EXPERIMENT

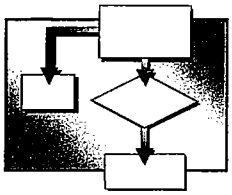
General Experimental Framework
Column
pH Map
Gradient/Elution Optimization
Loading Study



EVALUATE
- *Resolution*
- *Recovery*
- *Purification Table*
- *Capacity*
- *Practicality*

IMPLEMENT
- *Analytical Method Development*
- *Multi-step Separations*
- *Scale up*

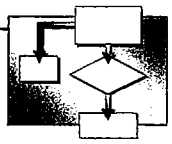
TROUBLESHOOT
- *Bandspreading*
- *Peak Shape*
- *Selectivity*
- *Recovery*
- *Pressure*
- *Reproducibility*
- *Column Cleaning and Reuse*
- *Cycle Time*



SECTION 2
PRINCIPLES OF SYSTEMATIC METHOD DEVELOPMENT

This section discusses the fundamental principles that you should follow in carrying out any chromatographic method development. It assumes that you have a good understanding of how chromatography works, which is covered in Section 5 — *Basics of Chromatography*. Specific approaches for different classes of biomolecules are discussed in Section 3 — *Developing Your Application*. Detailed method protocol recommendations are given in Section 4 — *Modes of Chromatography*.

Many users of chromatography, when developing a method using conventional media, have had to face very long experimental times, not only to set up and perform individual chromatographic runs, but also to analyze the resulting fractions. The length of the experiments inevitably limits the number of parameters that can be examined during the development process. This has forced many to resort to a kind of "hit and miss" approach, in which the chromatographic runs are viewed as trial solutions which either succeed or fail, rather than as useful data points about the behavior of the system. Often many parameters are changed at one time in moving from one trial solution to another, making it difficult to develop the kind of understanding that would allow an optimal method to be effectively designed and maintained.



With the advent of high speed Perfusion Chromatography media, individual run times (for both the chromatography itself and much of the analysis) have been sharply reduced. This makes it practical to take a more systematic approach, in which the critical parameters of the separation system are empirically examined, one at a time. The resulting information about the behavior of the system enables the user to actually design an optimal solution based on real data, as well as implement and maintain the method with a great deal of assurance.

Systematic method development may be viewed as a process with five stages, as follows:

Define	This is the critical stage in which you carefully delineate the problem to be solved, including the nature of the target molecule itself and the sample from which you are separating it, the analytical methods you will use, the overall goals of the separation, and the resources available.
Experiment	Once the problem is defined, you gather empirical information about the behavior of the system with respect to each of the key variables. Experimentation is carried out in a cycle of selecting systems of columns and mobile phases, then testing the effects of key parameters such as pH, gradient slope and, perhaps, sample load on each system.
Evaluate	Throughout the experimentation, you must continually evaluate the resolution, recovery, capacity and practicality of the method. The results of the evaluation are fed back into the design of the experimentation.
Implement	Based on the results of the experimental program and evaluation, you can then design your final method, optimize it and put it into practice. Details of the implementation vary, depending upon whether you are developing an analytical method, a multi-step separation or need to scale up.
Troubleshoot	Inevitably, you will be faced at some point with the need to solve problems with or fine tune the performance of your method. Chromatographic efficiency, selectivity, recovery, reproducibility, column equilibration, regeneration and reuse and method cycle time are all factors that might need optimization.

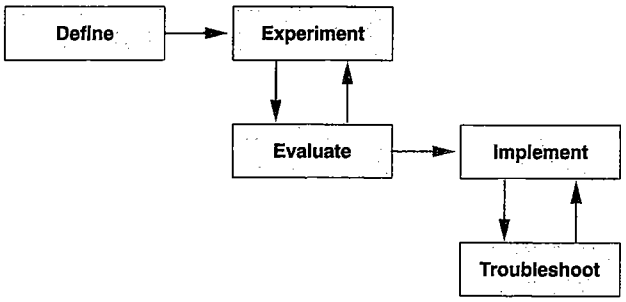


Figure 2-1. Stages of systematic chromatography method development

The following sections discuss in detail the considerations for each of these systematic method development stages.

DEFINE

In any field as complex as biomolecule chromatography, you must carefully define the problem you are trying to solve in order to be successful. You will not regret time spent at the beginning (*before* you go into the lab) examining the nature of the target molecule and sample source, choosing the analytical methods you will be using during the development, and evaluating the resources (time, equipment and sample) you will have available. Perhaps most importantly, you will need to clearly understand the goals or objectives of the separation (particularly whether it is analytical or preparative) and consider the specific method you are developing in the context of the overall separation strategy.

The Target Molecule & Sample

The starting point for defining a separation problem is obviously the target molecule and the sample from which it is to be separated. The class of molecule and molecular characteristics of the target relative to the other molecules in the sample