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P-2019/24831

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. **NC2019/0006822**
Solicitud de patente a nombre de **OMRIX BIOPHARMACEUTICALS LTD.**

Yo, CAROLINA DAZA MONTALVO identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, obrando como apoderada de la sociedad OMRIX BIOPHARMACEUTICALS LTD., domiciliada en Rehovot, Israel, solicitante de la patente de la referencia, en respuesta al oficio No. **11702** notificado electrónicamente el 26 de julio de 2021, relacionado con los requerimientos de fondo de la solicitud, atentamente me permito presentar la siguiente información:

1. Modificaciones a las Reivindicaciones

Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo capítulo reivindicatorio, el cual consta de nueve (9) reivindicaciones y reemplaza completamente el capítulo presentado anteriormente. Las modificaciones realizadas se presentan a continuación:

1.1 Se ha modificado la reivindicación 1, incluyendo las características de las antiguas reivindicaciones 2 y 3, y la expresión *"polianiones que son moléculas o complejos químicos que tienen más de una carga negativa"*, con el fin de dar

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claridad a la presente invención. Dicha modificación se encuentra soportada en la página 8 de la solicitud PCT.

1.2 Se ha eliminado el término *"opcionalmente"* del capítulo reivindicatorio de acuerdo con las sugerencias del examinador.

1.3 Se ha modificado la reivindicación 8 (nueva reivindicación 4) para recitar *"un polímero reticulado"*, con el fin de unificar los términos de acuerdo con las sugerencias del examinador.

1.4 Se han eliminado las antiguas reivindicaciones 4, 5, 14 y 15 de acuerdo con las modificaciones realizadas a la reivindicación 1.

1.5 Las reivindicaciones restantes, así como sus dependencias, fueron reenumeradas.

En cuanto a estas modificaciones, me permito decir que las mismas no amplían de ninguna manera la materia objeto de la presente solicitud y se realizaron con el ánimo de superar las objeciones de claridad y patentabilidad planteadas por el examinador en su concepto técnico.

2. Pago de tasas

En relación con el número de reivindicaciones, atentamente nos permitimos decir que no se adjunta tasa por concepto de reivindicaciones adicionales, toda vez que el nuevo capítulo reivindicatorio tiene un menor número de reivindicaciones a las presentadas anteriormente, y en esencia es el mismo objeto, pero definido con mayor claridad.

Por otra parte, me permito adjuntar recibo por concepto de pago de tasa de modificación a las reivindicaciones, según lo establecido en la **Resolución No. 59669 de 2020**.

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3. Claridad y concisión de las Reivindicaciones

El solicitante desea manifestar que mediante el nuevo capítulo reivindicatorio quedan subsanadas la totalidad de las objeciones, ya que las nuevas reivindicaciones proporcionan claridad y concisión a la invención reclamada, cuentan con pleno soporte en la descripción de la solicitud en estudio, y además siguen las recomendaciones realizadas por el examinador en su concepto técnico. En particular, frente a cada objeción, me permito señalar lo siguiente:

3.1 El examinador indica en el Oficio: *"Pese a las modificaciones presentadas, en capítulo reivindicatorio no cumple con el requisito de sustento en la descripción porque utiliza un lenguaje muy amplio que involucra múltiples posibilidades que no encuentran sustento técnico en la descripción. En este sentido se llama la atención del solicitante frente a las expresiones*

— Intercambiador de aniones
— Opcionalmente
— Portador farmacéuticamente aceptable
— Polianiones
— Grupos cargados positivamente
— Base fuerte, base débil
— Grupos amino cuaternarios, grupo amino primario, un grupo amino secundario, un grupo amino terciario

las cuales son imprecisas y definen específicamente el objeto reclamado, no hay en la descripción <u>sustento o evidencia técnica</u> que explique dicha generalización. Se le recomienda al solicitante que limite el alcance de la materia reclamada."

En este respecto y como se indicó previamente, se eliminaron las antiguas reivindicaciones 4, 5, 14 y 15. Se modificó la reivindicación 1 incluyendo las características de las antiguas reivindicaciones 2 y 3. También se eliminó el término *"opcionalmente"* del capítulo reivindicatorio, con el fin de dar claridad a la presente invención y superar las objeciones del examinador.

Ahora bien, el solicitante desea señalar que los términos *"Intercambiador de aniones", "grupos cargados positivamente", "base fuerte", "base débil"*, se definen específicamente en la página 2, líneas 29-30 y página 7, líneas 21-29

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de la solicitud PCT, y también son términos bien conocidos en la técnica, ver la tabla 1.2 presentada a continuación (ver la página 23 de Principios y Métodos de Cromatografía de Intercambio Iónico del documento adjunto a este memorial). Los polianiones se definen específicamente en la página 8 de la solicitud PCT, como moléculas o complejos químicos que tienen más de una carga negativa. Además, el término *"portador farmacéuticamente aceptable"* se define específicamente en la página 5, líneas 6-11 de la solicitud PCT.

Además, el Solicitante desea enfatizar que existe evidencia técnica en la descripción, ya que se ejemplifica más de un tipo de intercambiadores de aniones (como se define en la página 3), por ejemplo, intercambiadores de aniones que incluyen una base fuerte (es decir, QAE) e intercambiadores de aniones que incluyen una base débil (es decir, DEAE). Como se muestra en el Ejemplo 2, los intercambiadores de aniones tales como DEAE y QAE SEPHADEX™ unidos a una matriz junto con un ión calcio inducen la hemostasia. El ejemplo 2 muestra que el intercambiador de aniones unido a una matriz junto con una sal de calcio es esencial para la hemostasia.

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Functional groups

The functional groups substituted onto a chromatographic matrix (Table 1.2) determine the charge of an IEX medium, that is, a positively charged anion exchanger or a negatively charged cation exchanger.

Table 1.2. Functional groups used on ion exchangers

Anion exchangers		Functional group
Quaternary ammonium (Q)	strong	$-CH_2-N^+-(CH_3)_3$
Diethylaminoethyl (DEAE)*	weak	$-CH_2-CH_2-N^+-(CH_2-CH_3)_2$
Diethylaminopropyl (ANX)*	weak	$-CH_2-CHOH-CH_2-N^+-(CH_2-CH_3)_2$
Cation exchangers		**Functional group**
Sulfopropyl (SP)	strong	$-CH_2-CH_2-CH_2-SO_3^-$
Methyl sulfonate (S)	strong	$-CH_2-SO_3^-$
Carboxymethyl (CM)	weak	$-CH_2-COO^-$

* *The active end of the charged group is the same for DEAE and ANX. The difference between them is in the length of the carbon chain of the charged group. DEAE has a diethylaminoethyl group bound to the agarose. ANX has a diethylaminopropyl group attached which prevents the formation of quaternary groups, giving a different selectivity compared to DEAE.*

The terms strong and weak refer to the *extent that the ionization state of the functional groups varies with pH*. The terms strong and weak do not refer to the strength with which the functional groups bind to proteins. Strong ion exchangers show no variation in ion exchange capacity with change in pH (Fig 1.15). These exchangers do not take up or lose protons with changing pH and so have no buffering capacity, remaining fully charged over a broad pH range. Strong ion exchangers include Q (anionic), S, and SP (cationic).

3.2 El examinador ha indicado *"El capítulo reivindicatorio no cumple con el requisito de claridad porque la reivindicación 9 habla de un polímero reticulado que no había sido mencionado en las reivindicaciones precedentes, sírvase unificar los términos, refiriéndose bien sea a un polímero reticulado o a una matriz polimérica reticulada en todas las reivindicaciones".* En este respecto y como se indicó previamente, se ha modificado la antigua reivindicación 8 (nueva reivindicación 4) para recitar *"un polímero reticulado"*, con el fin de unificar los términos de acuerdo con las sugerencias del examinador. Por tanto, se solicita amablemente sea retirada la objeción.

3.3 Las reivindicaciones restantes, así como sus dependencias, fueron reenumeradas.

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En consecuencia, nos permitimos indicar que el presente capítulo reivindicatorio, que incluye nueve (9) reivindicaciones, se ajusta a las disposiciones del artículo 30 de la Decisión 486/00.

4. Nivel Inventivo

El examinador establece que las reivindicaciones 1 a 15 carecen de nivel inventivo a la luz de las enseñanzas descritas en los documentos WO2013/071235 (D1) y US5502042 (D3).

A este respecto, me permito decir que el artículo 18 de la Decisión 486 establece que *"Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica"*.

Lo anterior significa que el análisis de nivel inventivo **NO** es una comparación de los objetos de las anterioridades y la solicitud en estudio. El análisis de nivel inventivo **debe demostrar** la obviedad de la invención y establecer cómo ésta no contiene un salto cualitativo, que demuestre su altura inventiva, teniendo en cuenta que se hace **necesario demostrar** que la invención analizada carece de nivel inventivo y hubiese sido obvia para una persona versada en la materia.
De acuerdo con lo anterior, el solicitante desea manifestar respetuosamente su desacuerdo, ya que teniendo en cuenta las modificaciones presentadas en el nuevo capítulo reivindicatorio, se superan las objeciones realizadas, por cuanto **dichas características no se encuentran divulgadas individualmente en los documentos citados por el examinador**. Por tanto, se ve superada la carencia de nivel inventivo de la presente solicitud.

El examinador ha indicado: *"La diferencia entre la invención definida en la reivindicación 1 y la composición divulgada en D1 consiste en que está desprovista de polianiones. No obstante, no se observa un efecto técnico asociado a la diferencia. Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: ¿Cómo modificar la composición conocida en D1 con el fin de obtener una composición alternativa?".* En este

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respecto, el solicitante desea manifestar respetuosamente su desacuerdo por las siguientes razones:

Se sostiene que el problema técnico objetivo no debería formularse como la provisión de un material hemostático alternativo, sino más bien como la provisión de un material hemostático mejorado. D1 describe una composición polimérica biocompatible para el tratamiento de heridas y hemorragias que comprende un polímero hemofílico tal como DEAE y un polímero biocompatible (polianiónico) (ver, por ejemplo, el resumen de D1). Aunque en la presente solicitud no se proporciona una comparación directa con las composiciones de D1, los resultados incluidos en la solicitud demuestran, no obstante, una hemostasia mejorada en ausencia de cargas polianiónicas. Además, desde la página 23 hasta el último párrafo de la página 25, el primer párrafo de la solicitud tal como se presentó describe cómo un material con un grupo negativo en lugar de un grupo positivo no fue tan efectivo como una pinza hemostática. En particular, la composición con un grupo negativo (SP) no mostró ninguna reducción en el sangrado. Por el contrario, las composiciones equivalentes que utilizan un grupo DEAE o una amina cuaternaria como intercambiador de aniones y calcio dieron como resultado una reducción del índice de sangrado de 1 y 3, respectivamente.

La reivindicación 1 de la presente invención reclama una composición que comprende un intercambiador de aniones y una sal de calcio para su uso como medicamento para inducir la hemostasia en un sitio de hemorragia. El intercambiador de aniones y la sal de calcio se combinan en una composición, antes de que la composición se aplique al lugar del sangrado.

Por el contrario, según D1, el compuesto polimérico biocompatible (114) se reticula in situ aplicando una neblina de aerosol que comprende 0,1% a 1% en peso de cloruro de calcio, 1% a 5% en peso de nitrocelulosa y 94 % a 98,9% en peso de acetato de amilo. Como se ve, en D1, el intercambiador de aniones se aplica primero al sitio de sangrado, y luego se aplica la niebla de aerosol que comprende la sal de calcio para reticular el compuesto, de modo que el intercambiador de aniones y la sal de calcio se combinan en el sitio de sangrado.

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La composición de la reivindicación 1 pendiente se refiere a una composición que se puede fabricar en una industria, en lugar de una composición que se forma cuando los componentes se aplican por separado en el lugar del sangrado.

El examinador afirma que el *"documento D3 divulga composiciones para el tratamiento de heridas que incluyen un polisacárido reticulado oxidado que tiene una carga inducida químicamente. Los polisacáridos preferidos son los dextranos reticulados (Resumen)"*.

El examinador agrega:

"Sin embargo, composiciones hemostáticas para el tratamiento de heridas que estén desprovistas de polianiones ya están divulgadas en el documento 3, el cual se refiere a una composición que comprende: un tratamiento de heridas hidrolizable que incluye un polisacárido oxidado, en el que dicho polisacárido es una perla reticulada, particularmente dextrano; y en el que dicha perla de polisacárido reticulado tiene una carga inducida químicamente en la misma, donde la carga químicamente inducida es proporcionada por grupos dietilaminoetilo en el polisacárido (reivindicaciones). En los ejemplos se divulgan composiciones que comprenden DEAE SEPHADEX oxidadas."

Sin embargo, D3 no revela ni muestra una composición hemostática. Por el contrario, la composición de D3 se aplica para curar la herida <u>sólo</u> después de que se haya logrado la hemostasia mediante transferencia y/o presión (Ejemplo 15, líneas 34-37). Además, D3 no motiva ni menciona tener calcio en la composición.

El solicitante desea enfatizar que D1 requiere que un polianiónico y policatiónico se reticulen para formar una matriz gelatinosa incluso antes de su aplicación o antes de entrar en contacto con la sangre. Por ejemplo, D1 describe que la mezcla de polímeros polianiónicos con polímeros policatiónicos conduce a una reticulación iónica entre el "polímero hemofílico" ([0029]) (o polímero policatiónico, véase, por ejemplo, en la Figura 2, 224) y el "polímero estructural" (o polímero polianiónico, véase, por ejemplo, en la Figura 2, 226). Debido a esta reticulación, la composición polimérica funciona como una composición

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gelatinosa de matriz hemostática y cicatrizante de heridas que puede formarse interna o externamente ([0013], [0017]). Por tanto, no hay motivación en D1 para eliminar polianiones como en D3, ya que hacerlo dará como resultado un componente no gelatinoso necesario para la hemostasia de acuerdo con D1.

Por el contrario, la presente reivindicación menciona una composición que "carece de polianiones". Sorprendentemente, se encontró que una composición como en la presente reivindicación desprovista de polianiones (como se muestra en todos los ejemplos) y que comprende calcio induce la hemostasia.

Por tanto, D1 en combinación con D3 no motiva la composición reclamada. En vista de lo anterior, las presentes reivindicaciones son inventivas a la luz de D1 y D3 solas o en conjunto.

De esta forma, teniendo en cuenta que los documentos D1 y D3 **no mencionan, describen o sugieren**, en forma individual o en combinación, todas las características técnicas reclamadas como se describe en la presente invención, **dichas reivindicaciones son novedosas, e inventivas sobre el estado del arte**.

En este sentido, y siguiendo el Manual Andino de Patentes, la presente invención tal y como se encuentra definida en las reivindicaciones 1 a 9, tiene diferencias con el estado del arte e implicó un nivel inventivo durante su desarrollo, por lo cual debe ser considerada como novedosa e inventiva según las disposiciones de los artículos 16 y 18 de la Decisión 486.

5. Conclusión

Con las modificaciones introducidas y los argumentos presentados puede concluirse que:

i) La presente solicitud es clara, concisa y está debidamente soportada en la descripción, cumpliendo con los requerimientos del Artículo 30 de la Decisión 486; y

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ii) Las reivindicaciones actuales son patentables sobre D1 y D3, cumpliendo así con los requerimientos del Artículo 18 de la Decisión 486.

Por tanto, atentamente me permito solicitar a ese Despacho otorgar el privilegio de patente solicitado a la solicitud en estudio, toda vez que cumple a cabalidad con los requerimientos de la Decisión 486/00.

En consecuencia, solicitamos a ese Despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional.

6. Anexos

- Nuevo capítulo reivindicatorio conformado por 9 reivindicaciones.
- Principios y Métodos de Cromatografía de Intercambio Iónico.
- Recibo de pago correspondiente a la Tasa de modificaciones a las reivindicaciones.

Señora Directora,

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REIVINDICACIONES MODIFICADAS

1. Una composición hemostática que comprende:

un intercambiador de aniones en donde el intercambiador de aniones comprende uno o más grupos cargados positivamente unidos a una matriz, en donde los grupos cargados positivamente consisten en una base seleccionada del grupo que consiste en una base fuerte, una base débil y una combinación de estas;

una sal de calcio; y

un portador farmacéuticamente aceptable;

en donde la composición hemostática está desprovista de polianiones que son moléculas o complejos químicos que tienen más de una carga negativa.

2. La composición hemostática de acuerdo con la reivindicación 1, en donde la base débil consiste en grupos dietilaminoetilo (DEAE).

3. La composición hemostática de acuerdo con una cualquiera de las reivindicaciones 1 a 2, en donde la matriz se selecciona del grupo que consiste en un poliéster alifático, un polisacárido, un polipéptido, poliestireno-divinilbenceno, sílice y una combinación de estos.

4. La composición hemostática de acuerdo con una cualquiera de las reivindicaciones 1 a 3, en donde la matriz comprende un polímero reticulado.

5. La composición hemostática de acuerdo con la reivindicación 4, en donde el polímero reticulado está covalentemente reticulado.

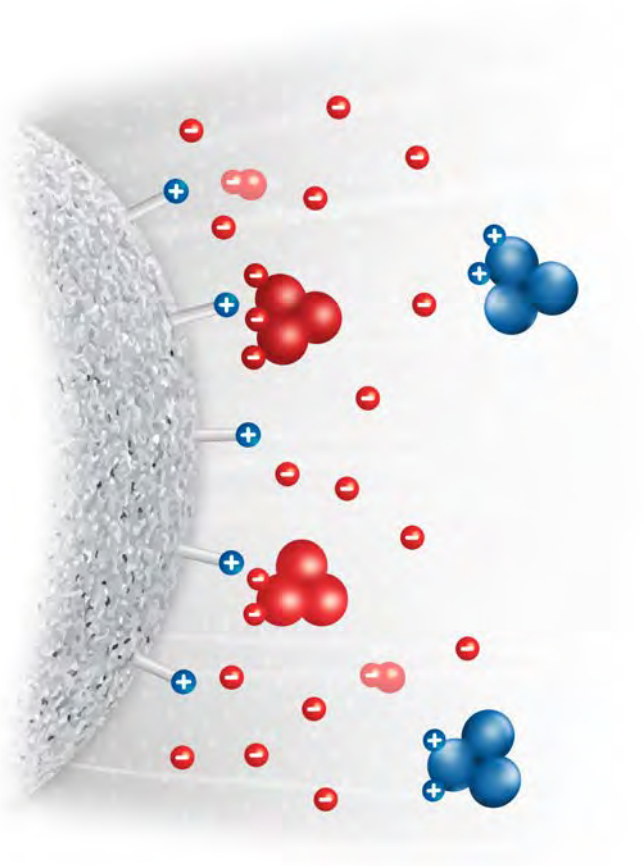
6. La composición hemostática de acuerdo con una cualquiera de las reivindicaciones 3 a 5, en donde el polisacárido se selecciona del grupo que consiste en celulosa, dextrano, agarosa y combinaciones de estos.

7. La composición hemostática de acuerdo con una cualquiera de las reivindicaciones 1 a 6, en donde la composición está sustancialmente desprovista de cualquier proteína de la cascada de coagulación sanguínea.

8. La composición hemostática de acuerdo con una cualquiera de las reivindicaciones 1 a 7, en donde la composición está en una forma seleccionada del grupo que consiste en una suspensión, polvo, película, parche y líquido.

9. La composición hemostática de acuerdo con la reivindicación 8, en donde la composición en la forma de suspensión o líquido comprende, además, un portador farmacéuticamente aceptable.

GE Healthcare

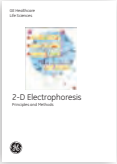


Ion Exchange Chromatography

Principles and Methods

Handbooks from GE Healthcare Life Sciences

For more information refer to www.gelifesciences.com/handbooks



2-D Electrophoresis using Immobilized pH Gradients
Principles and Methods
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GST Gene Fusion System
Handbook
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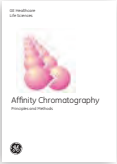
Nucleic Acid Sample Preparation for Downstream Analyses
Principles and Methods
28962400



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High-throughput Process Development with PreDictor Plates
Principles and Methods
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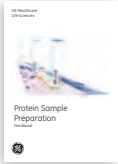

Protein Sample Preparation
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ÄKTA Laboratory-scale Chromatography Systems
Instrument Management Handbook
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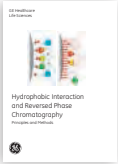
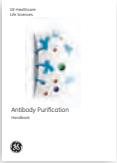


Hydrophobic Interaction and Reversed Phase Chromatography
Principles and Methods
11001269

Purifying Challenging Proteins
Principles and Methods
28909531



Antibody Purification
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18103746

Imaging
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29020301

Recombinant Protein Purification Handbook
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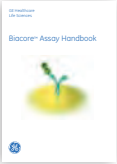
18114275



Biacore Assay
Handbook
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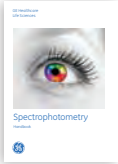
11000421



Spectrophotometry
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29033182

Biacore Sensor Surface
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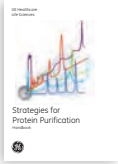

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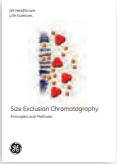

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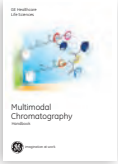

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Handbook
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Ion Exchange Chromatography

Principles and Methods

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Introduction

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific properties, as shown in Figure I.1. Ion exchange chromatography (IEX) separates biomolecules according to differences in their net surface charge.

Property	Technique
Charge	Ion exchange chromatography (IEX)
Size	Size exclusion chromatography (SEC), also called gel filtration (GF)
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC)
Biorecognition (ligand specificity)	Affinity chromatography (AC)

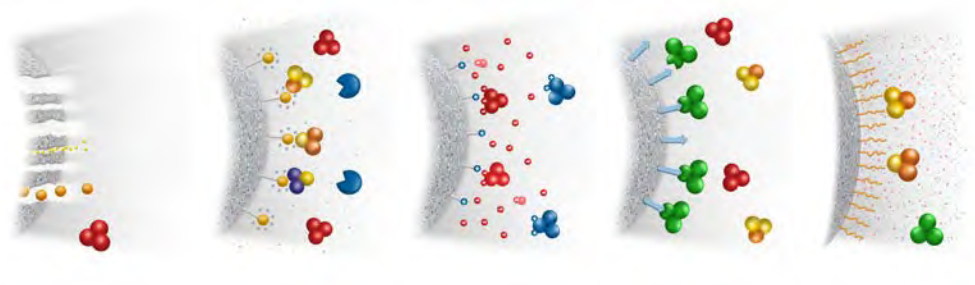


Fig I.1. Separation principles in chromatographic purification.

IEX for the separation of biomolecules was introduced in the 1960s and continues to play a major role in the separation and purification of biomolecules. Today, IEX is one of the most frequently used techniques for purification of proteins, peptides, nucleic acids, and other charged biomolecules, offering high resolution and group separations with high loading capacity. The technique is capable of separating molecular species that have only minor differences in their charge properties, for example two proteins differing by one charged amino acid. These features make IEX well suited for capture, intermediate purification, or polishing steps in a purification protocol and the technique is used from microscale purification and analysis through to purification of kilograms of product.

This handbook describes both theoretical and practical aspects principles of the technique, the chromatography media (resins) available and how to select them, application examples, and detailed instructions for the most commonly performed procedures. Practical information, with many tips and hints drawn from over 50 yr of experience in chromatography purification, guides beginners and experts towards obtaining optimal results from the latest chromatography media.

GE Healthcare's Life Sciences business offers a wide variety of prepacked columns and ready-to-use chromatography media. A range of handbooks ensure that purification with any chromatographic technique becomes a simple and efficient procedure at most scales and in most laboratories.

Symbols

This symbol indicates general advice on how to improve procedures or recommends measures to take in specific situations

This symbol indicates where special care should be taken

Highlights chemicals, buffers, and equipment

Outline of experimental protocol

Common acronyms and abbreviations

A_{280}	UV absorbance at specified wavelength (in this example, 280 nm)
AC	affinity chromatography
AIEX	anion exchange chromatography
APMSF	4-aminophenyl-methylsulfonyl fluoride
AU	absorbance units
BSA	bovine serum albumin
cGMP	current good manufacturing practice
CF	chromatofocusing
CHO	Chinese hamster ovary
CIEX	cation exchange chromatography
CIP	cleaning-in-place
CIPP	capture, intermediate purification, polishing
CV	column volume
Dab	domain antibody, the smallest functional entity of an antibody
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DOC	deoxycholate
DoE	design of experiments
DS	desalting (group separation by size exclusion chromatography; buffer exchange)
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol-O,O'-bis-[2-amino-ethyl]-N,N,N',N',-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
$F(ab')_2$ fragment	fragment with two antigen binding sites, obtained by pepsin digestion
Fab fragment	antigen binding fragment obtained by papain digestion
Fc fragment	crystallizable fragment obtained by papain digestion
Fv fragment	unstable fragment containing the antigen binding domain
GF	gel filtration; also called size exclusion chromatography
GST	glutathione S-transferase

HCP	host cell protein
HIC	hydrophobic interaction chromatography
HMW	high molecular weight
HSA	human serum albumin
IEX	ion exchange chromatography
IgA, IgG etc.	different classes of immunoglobulin
IMAC	Immobilized metal ion affinity chromatography
LC-MS	liquid chromatography–mass spectrometry
LMW	low molecular weight
MAb	monoclonal antibody
MPa	megaPascal
M_r	relative molecular weight
MS	mass spectrometry
n	native, as in nProtein A
NC	nitrocellulose
NHS	N-hydroxysuccinimide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pI	isoelectric point, the pH at which a protein has zero net surface charge
PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
PVDF	polyvinylidene fluoride
PVP	polyvinylpyrrolidine
r	recombinant, as in rProtein A
RNA	ribonucleic acid
RNAse	ribonuclease
RPC	reversed phase chromatography
scFv	single chain Fv fragment
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
TCEP	tris(2-carboxyethyl) phosphine hydrochloride
TFA	Trifluoroacetic acid
Tris	tris-(hydroxymethyl)-aminomethane
UV	ultraviolet
v/v	volume to volume
w/v	weight to volume

Chapter 1
Principles of ion exchange

This chapter provides a general introduction to the theoretical principles that underlie every ion exchange separation. An understanding of these principles will enable the separation power of ion exchange chromatography (IEX) to be fully appreciated. Practical aspects of performing a separation are covered in Chapter 2.

Net surface charge and pH

IEX separates molecules on the basis of differences in their *net surface charge*. Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography media according to differences in their overall charge, charge density, and surface charge distribution. The charged groups within a molecule that contribute to the net surface charge possess different *pKa* values (acid ionization constant) depending on their structure and chemical microenvironment.

Since all molecules with ionizable groups can be titrated, their net surface charge is highly pH dependent. In the case of proteins, which are built up of many different amino acids containing weak acidic and basic groups, net surface charge will change gradually as the pH of the environment changes, that is, proteins are *amphoteric*. Each protein has its own unique *net charge versus pH relationship* which can be visualized as a *titration curve*. This curve reflects how the overall net charge of the protein changes according to the pH of the surroundings. Figure 1.1 illustrates several theoretical protein titration curves (these curves can be generated using a combination of isoelectric focusing and electrophoresis, but with modern solutions for rapid method development, actual titration curves are rarely used).

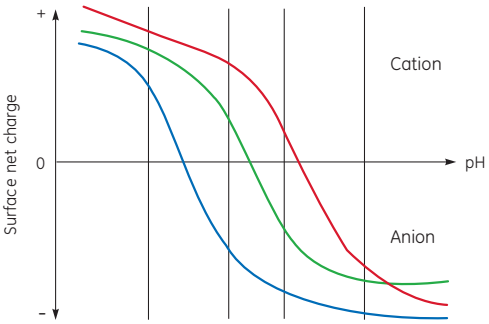


Fig 1.1. Theoretical protein titration curves, showing how net surface charge varies with pH.

IEX takes advantage of the fact that the relationship between net surface charge and pH is unique for a specific protein. In an IEX separation, *reversible interactions* between *charged* molecules and *oppositely charged* IEX media are controlled in order to favor binding or elution of specific molecules and achieve separation. A protein that has no net charge at a pH equivalent to its *isoelectric point (pI)* will not interact with a charged medium. However, at a pH above its pI, a protein will bind to a positively charged medium or *anion exchanger* and, at a pH below its pI, a protein will bind to a negatively charged medium or *cation exchanger*. In addition to the ion-exchange interaction, other types of binding can occur, but these effects are very small and mainly due to van der Waals forces and nonpolar interactions.

Steps in an IEX separation

An IEX medium comprises a matrix of spherical particles substituted with ionic groups that are negatively or positively charged. The matrix is usually porous to give a high internal surface area. The medium is packed into a column to form a packed bed. The bed is then equilibrated with buffer which fills the pores of the matrix and the space in between the particles.

Equilibration

The first step is the equilibration of the stationary phase to the desired start conditions. When equilibrium is reached, all stationary phase charged groups are bound with exchangeable counterions, such as chloride or sodium. The pH and ionic strength of the start buffer are selected to ensure that, when sample is loaded, proteins of interest bind to the medium and as many impurities as possible do not bind.

Sample application and wash

The second step is sample application and wash. The goal in this step is to bind the target molecule(s) and wash out all unbound material. The sample buffer should have the same pH and ionic strength as the start buffer in order to bind all charged target proteins. Oppositely charged proteins bind to ionic groups of the IEX medium, becoming concentrated on the column. Uncharged proteins, or those with the same charge as the ionic group, pass through the column at the same speed as the flow of buffer, eluting during or just after sample application, depending on the total volume of sample loaded.

Elution

When all the sample has been loaded and the column washed with start buffer so that all nonbinding proteins have passed through the column, conditions are altered in order to elute the bound proteins. Most frequently, proteins are eluted by increasing the ionic strength (salt concentration) of the buffer or, occasionally, by changing the pH. As ionic strength increases the salt ions (typically Na^+ or Cl^-) compete with the bound components for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column. The proteins with the lowest net charge at the selected pH will be the first ones eluted from the column as ionic strength increases. Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and will be eluted last. The higher the net charge of the protein, the higher the ionic strength that is needed for elution. By controlling changes in ionic strength using different forms of gradient, proteins are eluted differently in a purified, concentrated form.

Regeneration

A final wash with high ionic strength buffer regenerates the column and removes any molecules still bound. This ensures that the full capacity of the stationary phase is available for the next run. The column is then re-equilibrated in start buffer before starting the next run.

The above describes a typical IEX separation. Alternatively, conditions can be chosen to maximize the binding of contaminants to allow the target protein(s) to first pass through the column to be collected.

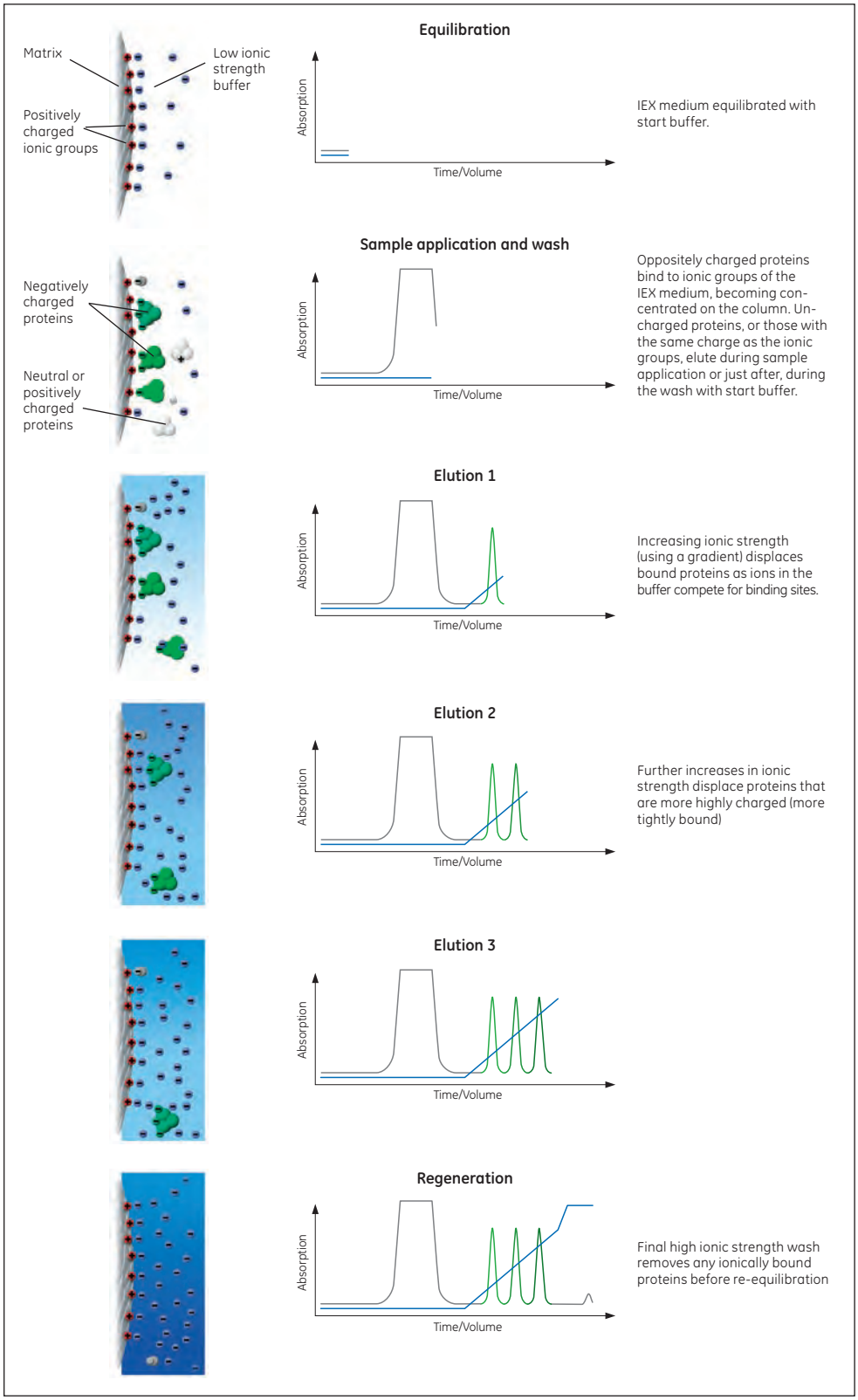


Fig 1.2. Principles of an anion exchange separation.

Resolution

The resolution of an IEX separation is a combination of the degree of separation between the peaks eluted from the column (the selectivity of the medium), the ability of the column to produce narrow, symmetrical peaks (efficiency) and, of course, the amount (mass) of sample applied. These factors are influenced by practical issues such as matrix properties, binding and elution conditions, column packing, and flow rates which are covered in detail in Chapter 2, *Ion exchange in practice*.

Resolution (R_s) is defined as the distance between peak maxima compared with the average base width of the two peaks. R_s can be determined from a chromatogram, as shown in Figure 1.3.

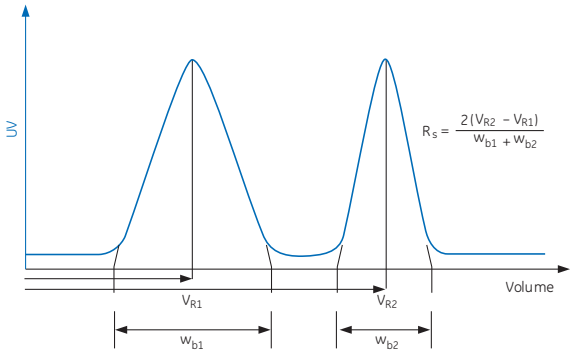


Fig 1.3. Determination of the resolution (R_s) between two peaks.

Elution volumes and peak widths are measured with the same units to give a dimensionless resolution value. R_s gives a measure of the relative separation between two peaks and can be used to determine if further optimization of the chromatographic procedure is necessary. If R_s = 1.0 (Fig 1.4) then 98% purity has been achieved at 98% of peak recovery, provided the peaks are symmetrical and approximately equal in size. Baseline resolution requires that $R_s \geq 1.5$. At this value, peak purity is 100%.

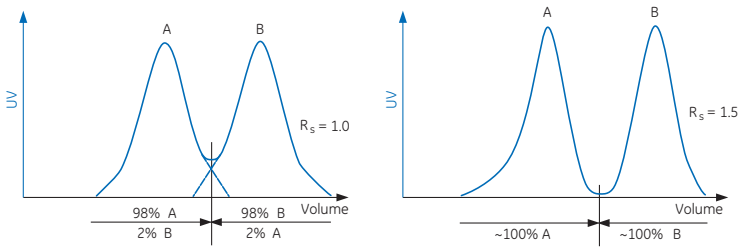


Fig 1.4. Separation results with different resolutions.

A single, well-resolved peak is not necessarily a pure substance, but might represent a series of components that could not be separated under the chosen elution conditions.

Efficiency

Column efficiency (the ability to elute narrow, symmetrical peaks from a packed bed) relates to the *zone broadening* which occurs on the column and is frequently stated in terms of the number of theoretical plates (see Appendix 3 for determination of column efficiency). One of the main causes of zone broadening is longitudinal diffusion of the solute molecules, that is, proteins, peptides, or oligonucleotides. Zone broadening can be minimized if the distances available for diffusion are minimized. In all situations, a well-packed column will contribute significantly to resolution. Columns that are packed unevenly, too tightly, too loosely, or that contain air bubbles will lead to channeling (uneven passage of buffer through the column), zone broadening and hence loss of resolution. Figure 1.5 illustrates the parameters that contribute to good column efficiency. Obviously particle size is a significant factor in resolution and, in general, the smallest particles will produce the narrowest peaks under the correct elution conditions and in a well-packed column.

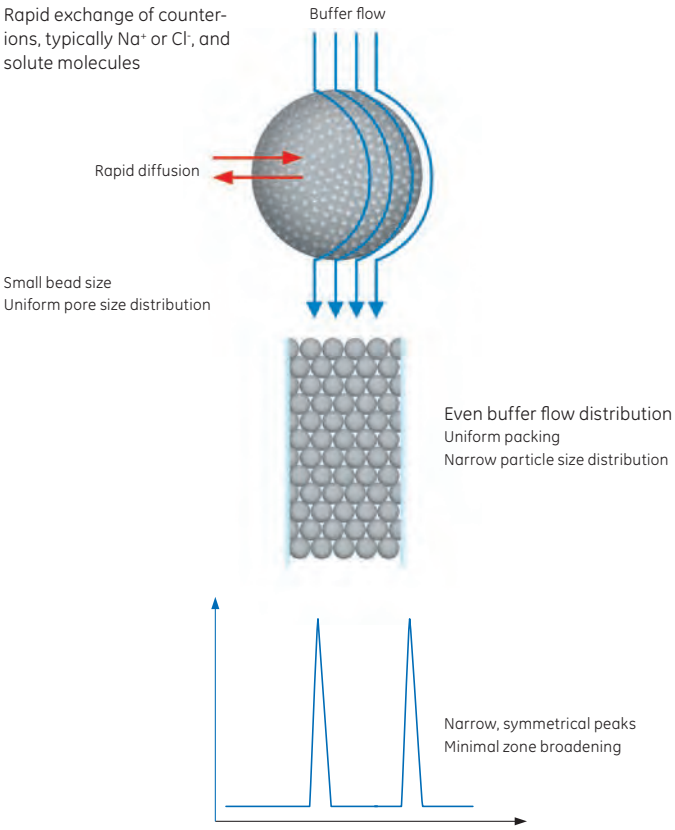


Fig 1.5. Factors that affect column efficiency.

Figure 1.6 demonstrates the influence of particle size on efficiency by comparing several different IEX media under exactly the same running conditions. Note that different media selectivities also influence the final resolution.

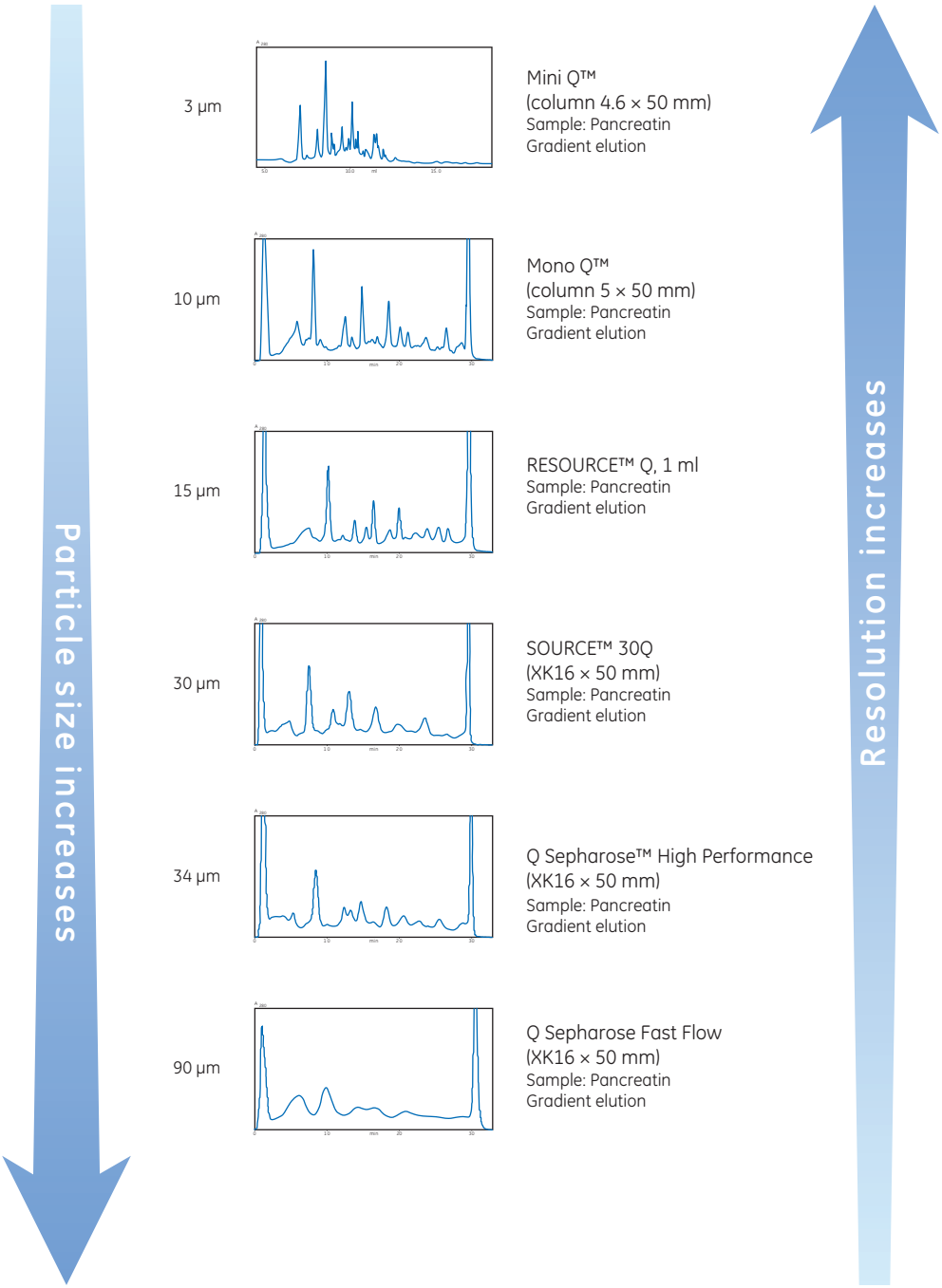


Fig 1.6. Examples of the influence of particle size and selectivity on final resolution.

Although resolution in terms of efficiency can be improved by decreasing the particle size of the matrix, using a smaller particle size often creates an increase in back pressure so that flow rates need to be decreased, lengthening the run time. Hence the need to match the medium with the requirements for the purification (speed, resolution, recovery, and capacity).

The viscosity of highly concentrated samples might reduce resolution if large sample volumes are loaded onto columns packed with small particles. Samples may be diluted or, alternatively, a larger particle size should be used.

Selectivity

Good selectivity (the degree of separation between peaks) is a more important factor than high efficiency in determining resolution (Fig 1.7) and depends not only on the nature and number of the functional groups on the matrix, but also on the experimental conditions, such as pH (influencing the protein charge), ionic strength, and elution conditions. It is the ease and predictability with which these experimental conditions can be manipulated, when using a suitably designed chromatography medium, that gives IEX the potential of extremely high resolution.

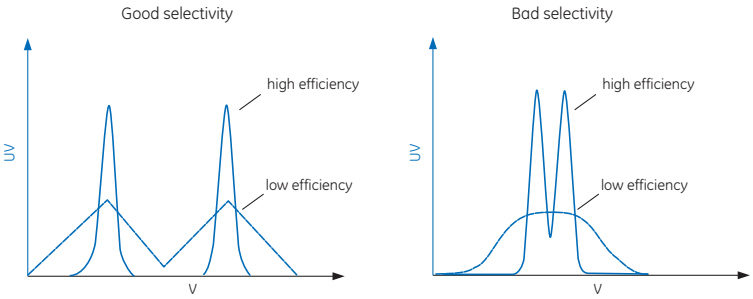


Fig 1.7. Effect of selectivity and efficiency on resolution.

Selectivity and pH

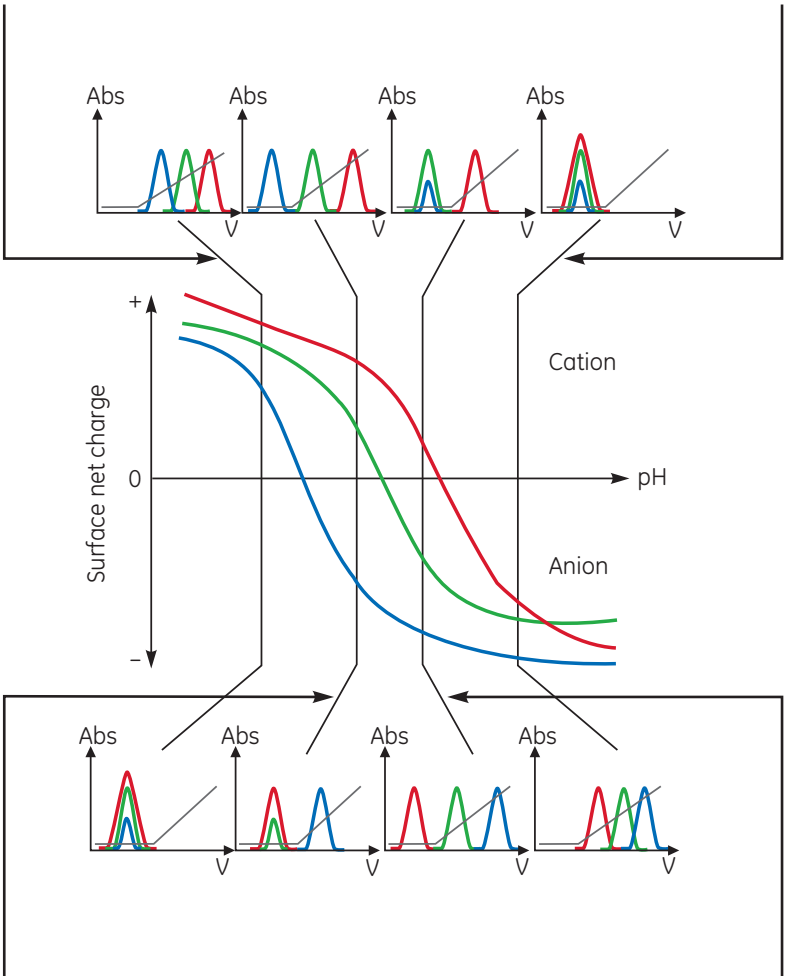
Good selectivity is achieved by performing IEX separations at pH values carefully selected to maximize the differences in net charge of the components of interest. Figure 1.8 emphasizes the significance of pH.

Optimum selectivity can be expected at a pH where there is maximum separation between the titration curves for the individual proteins (i.e., the difference in net charges between the species is greatest) and when using an ion exchanger with a charge opposite to the charge of the proteins at the particular pH.

The order in which proteins are eluted cannot always be predicted with absolute certainty since a titration curve (produced in practice by measuring electrophoretic mobility in a gel) reflects the total net charge on a protein and IEX depends on the net charge on the surface of the protein.

Most acidic pH: all three proteins are below their pI, positively charged, and bind only to a cation exchanger. Proteins are eluted in the order of their net charge.

Most alkaline pH: all three proteins are above their pI, negatively charged, and bind only to the anion exchanger. Proteins are eluted in the order of their net charge.



Less acidic pH: blue protein is above its pI, negatively charged, other proteins are still positively charged. Blue protein binds to an anion exchanger and can be separated from the other proteins which wash through. Alternatively, red and green proteins can be separated on a cation exchanger and the blue protein washes through.

Less alkali pH: red protein below its pI, positively charged. Red protein binds to cation exchanger and can be separated from the other proteins which wash through. Alternatively, blue and green proteins can be separated on an anion exchanger and the red protein washes through.

Fig 1.8. Effect of pH on protein binding and elution patterns.

Selectivity and elution

The figures below illustrate the most common forms of IEX separation in which proteins are eluted by increasing the ionic strength of a buffer (typically with NaCl) using *linear gradient or step elution*. The UV absorbance and conductivity traces show the elution of protein peaks and the changes in salt concentration, respectively, during elution.

Buffer volumes used during sample application, elution, washing and re-equilibration are expressed in *column volumes (CV)*, for example 5 CV = 5 ml for a column with a 1 ml bed volume. Using CV to describe a separation profile facilitates method development and transfer of methods to columns of different dimensions when scaling-up.

Gradient elution (Fig 1.9) is often used when starting with an unknown sample (as many components as possible are bound to the column and eluted differentially to see a total protein profile) and for high-resolution separation or analysis.

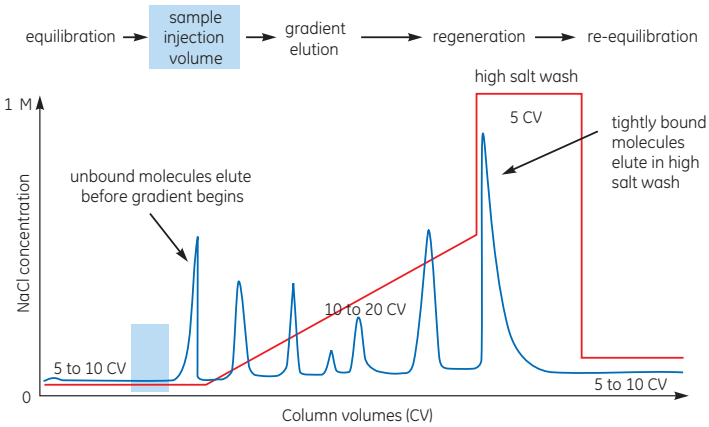


Fig 1.9. Typical high-resolution IEX separation using linear gradient elution.

Step elution is used in several ways. When an IEX separation has been optimized using gradient elution, changing to a step elution *speeds up separation times and reduces buffer consumption* while retaining the required purity level (Fig 1.10).

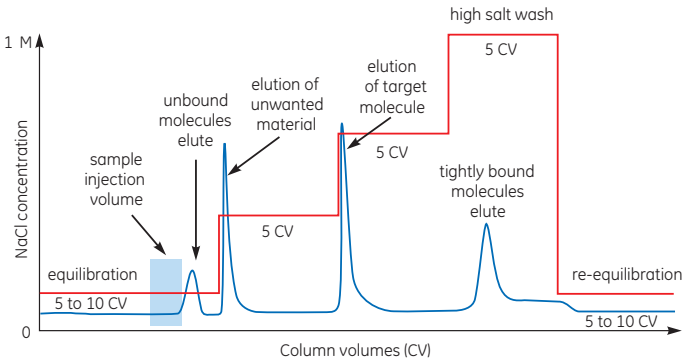


Fig 1.10. Typical IEX separation using step elution.

Step elution can also be used for *group separation* in order to concentrate the proteins of interest and rapidly remove them from unwanted substances (Fig 1.11). The target protein(s) is eluted in an enriched, concentrated form.

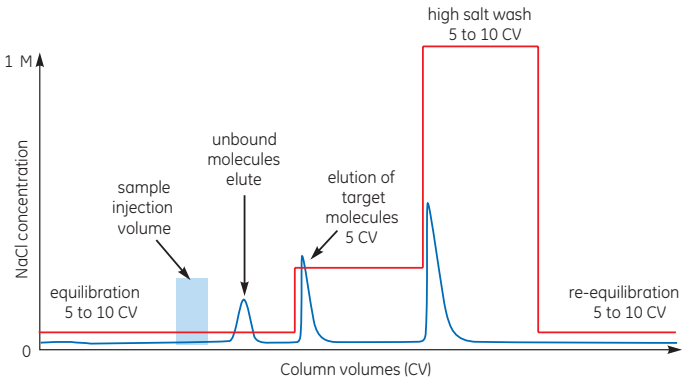


Fig 1.11. Typical IEX separation using a step elution to separate groups of proteins with very different charge properties.

Occasionally, step elution is used to *remove contaminants* by choosing conditions that maximize binding of the contaminants and allow the target protein(s) to pass through the column (Fig 1.12). Care must be taken to ensure that the binding capacity of the column is sufficient to bind all contaminants.

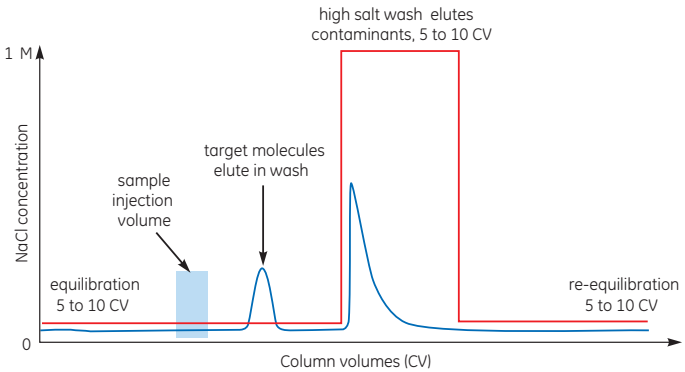


Fig 1.12. Contaminant removal: target protein(s) elute in the wash, contaminants bind to the column.

Components of IEX media

Chromatography media for ion exchange are made from porous or nonporous matrices, chosen for their physical stability, their chemical resistance to stringent cleaning conditions, and their low level of nonspecific interaction. The matrices are substituted with functional groups that determine the charge of the medium.

Matrix

- High porosity offers a large surface area covered by charged groups and so ensures a high binding capacity. High porosity is also an advantage when separating large biomolecules. Nonporous matrices are preferable for extremely high-resolution separations when diffusion effects must be avoided.
- An inert matrix minimizes nonspecific interactions with sample components.
- High physical stability ensures that the volume of the packed medium remains constant despite extreme changes in salt concentration or pH thus improving reproducibility and avoiding the need to repack columns.
- High physical stability and uniformity of particle size facilitate high flow rates, particularly during cleaning or re-equilibration steps, to improve throughput and productivity.
- High chemical stability ensures that the matrix can be cleaned using stringent cleaning solutions if required.
- Modern IEX media use either polymer or agarose-based matrices to fulfil not only the requirements for high binding capacity, chemical and physical stability, but to generate media with suitable particle sizes for a range of applications (Table 1.1).

Table 1.1. Ion exchange matrices

	Form	Mean particle size (µm)
MiniBeads™	Polystyrene/divinyl benzene	3
MonoBeads™	Polystyrene/divinyl benzene	10
SOURCE 15	Polystyrene/divinyl benzene	15
SOURCE 30	Polystyrene/divinyl benzene	30
Sepharose High Performance	Agarose 6%	34
Sepharose Fast Flow	Agarose 6%	90
Sepharose 4 Fast Flow	Agarose 4%	90
Sepharose XL	Agarose 6%, dextran chains coupled to agarose	90
Sepharose Big Beads	Agarose 6%	200
Capto™ ImpRes	High-flow agarose	40
Capto ImpAct	High-flow agarose	50
Capto	High-flow agarose	90

MiniBeads is a matrix made from polystyrene, with divinyl benzene as cross-linker, to produce highly spherical (monodispersed), very small (3 µm), nonporous particles that facilitate micropreparative or analytical separations when *extremely high resolution* is more important than high binding capacity or high flow rates.

MonoBeads and SOURCE are matrices made from polystyrene with divinyl benzene to produce highly spherical (monodispersed), small (10, 15, or 30 µm), porous particles (Fig 1.13) that facilitate *high resolution separations at high flow rates*.

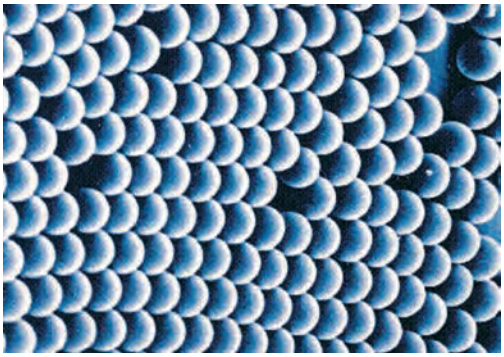


Fig 1.13. Electron micrograph of MonoBeads showing spherical, monodispersed particles.

Sepharose media are based on chains of agarose, arranged in bundles and with different degrees of cross-linking (Fig 1.14), to give a range of rigid, macroporous matrices with good capacity and low nonspecific adsorption. The most suitable matrix can be selected according to the degree of resolution, binding capacity, and flow rates desired for the separation. For example, gradient elution on Sepharose High Performance (34 µm) will give a high-resolution separation whereas the larger particles of Sepharose Fast Flow (90 µm) or Sepharose Big Beads (200 µm) would be most suited to high-capacity step elution at high flow rate.

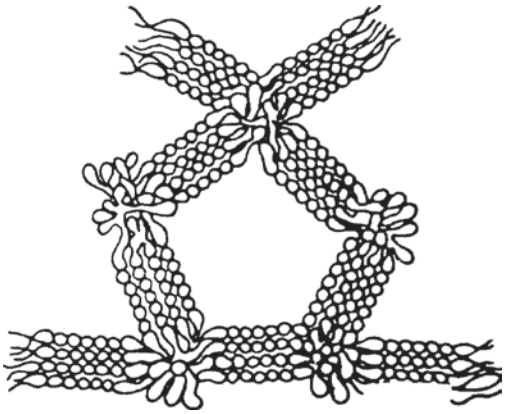


Fig 1.14. Structure of cross-linked agarose media (Sepharose).

Capto media are based on a chemically modified, high-flow agarose matrix. This matrix provides particle rigidity without compromising pore size, outstanding pressure/flow properties, and high chemical stability to support CIP procedures. Capto media are suitable for scaling up and for use in large-scale bioprocess purifications. The basic characteristics of Capto (90 µm), Capto ImpAct (50 µm), and Capto ImpRes (40 µm) IEX media are summarized in Chapter 3.

Functional groups

The functional groups substituted onto a chromatographic matrix (Table 1.2) determine the charge of an IEX medium, that is, a positively charged anion exchanger or a negatively charged cation exchanger.

Table 1.2. Functional groups used on ion exchangers

Anion exchangers		**Functional group**
Quaternary ammonium (Q)	strong	$-CH_2-N^+-(CH_3)_3$
Diethylaminoethyl (DEAE)*	weak	$-CH_2-CH_2-N^+-(CH_2-CH_3)_2$
Diethylaminopropyl (ANX)*	weak	$-CH_2-CHOH-CH_2-N^+-(CH_2-CH_3)_2$
Cation exchangers		**Functional group**
Sulfopropyl (SP)	strong	$-CH_2-CH_2-CH_2-SO_3^-$
Methyl sulfonate (S)	strong	$-CH_2-SO_3^-$
Carboxymethyl (CM)	weak	$-CH_2-COO^-$

* *The active end of the charged group is the same for DEAE and ANX. The difference between them is in the length of the carbon chain of the charged group. DEAE has a diethylaminoethyl group bound to the agarose. ANX has a diethylaminopropyl group attached which prevents the formation of quaternary groups, giving a different selectivity compared to DEAE.*

The terms strong and weak refer to the *extent that the ionization state of the functional groups varies with pH*. The terms strong and weak do not refer to the strength with which the functional groups bind to proteins. Strong ion exchangers show no variation in ion exchange capacity with change in pH (Fig 1.15). These exchangers do not take up or lose protons with changing pH and so have no buffering capacity, remaining fully charged over a broad pH range. Strong ion exchangers include Q (anionic), S, and SP (cationic).

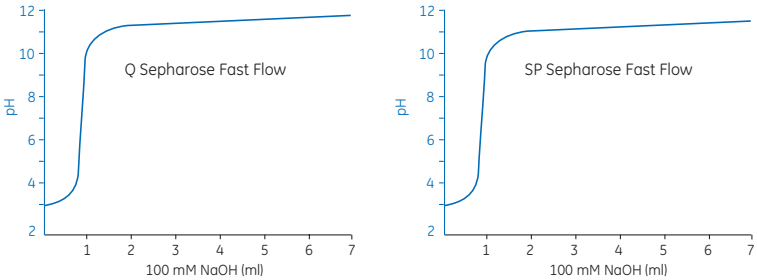


Fig 1.15. Titration curves show the ion exchange capacity of strong ion exchangers Q and SP. Approximately 5 ml of Q or SP Sepharose Fast Flow were equilibrated in 1 M KCl and titrated with 100 mM NaOH.

There are several advantages to working with strong ion exchangers:

- Development and optimization of separations is fast and easy since the charge characteristics of the medium do not change with pH.
- The mechanism of interaction is simple since there are no intermediate forms of charge interaction.
- Sample loading (binding) capacity is maintained at high or low pH since there is no loss of charge from the ion exchanger.

The majority of proteins have pI within the range 5.5 to 7.5 and can be separated on either strong or weak ion exchangers. An advantage of a weak ion exchanger, such as DEAE (anionic), ANX (anionic), and CM (cationic) is that they can offer a different selectivity compared to strong ion exchangers. A disadvantage is that because weak ion exchangers can take up or lose protons with changing pH, their ion exchange capacity varies with pH (Fig 1.16).

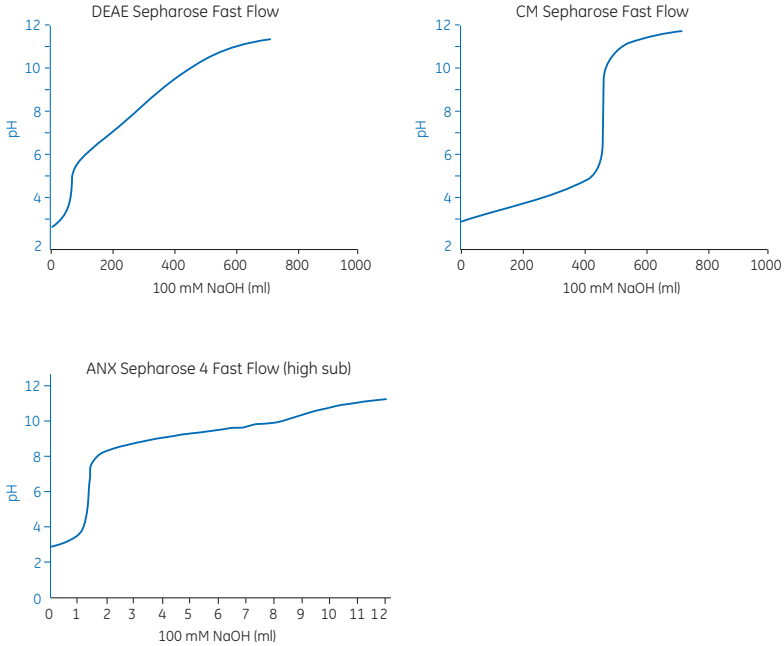


Fig 1.16. Titration curves show how the ion exchange capacity of weak ion exchangers varies with pH.

Try a weak ion exchanger such as DEAE, CM, or ANX Sepharose Fast Flow, if a strong ion exchanger (substituted with Q, S, or SP) does not give the required selectivity.

Binding capacity and recovery

The capacity of an IEX medium is a quantitative measure of its ability to take up counterions (proteins or other charged molecules). The *total ionic capacity* is the number of charged functional groups/ml medium, a fixed parameter of each medium. Of more practical relevance is the actual amount of protein that can bind to an IEX medium, under defined experimental conditions. The capacity of a chromatography medium can be measured in two ways: static binding capacity (SBC) and dynamic binding capacity (DBC). SBC is the maximum amount of protein that can bind at given conditions. SBC is often obtained during excess load of sample. DBC is measured during given conditions, including flow rate, and is the amount of protein that binds before a significant breakthrough of the target protein appears. Figures for binding capacity in this handbook refer to the dynamic binding capacity.

The static and dynamic binding capacities depend upon the properties of the protein, the IEX medium, and the experimental conditions. The capacity of an IEX medium will vary according to the molecular size of the specific protein (which affects its ability to enter all the pores of the matrix) and its charge/pH relationship (the protein must carry the correct net charge at a sufficient surface density at the chosen pH). With earlier IEX media, larger biomolecules had limited access to the functional groups, significantly reducing the binding capacity.

Nowadays, ion exchange matrices such as MonoBeads, Capto, SOURCE, and Sepharose media all have exclusion limits for globular proteins in excess of 1×10^6 and are therefore suitable for the majority of biomolecule separations. Binding capacities will still vary according to the molecular size of the biomolecules. For example, a matrix with a high degree of small pores will exhibit a higher binding capacity for smaller molecules. Experimental conditions such as pH, ionic strength, counterion, flow rate, and temperature should all be considered when comparing binding capacities of different IEX media.

Modern IEX media show very low levels of nonspecific adsorption so that sample recovery under suitable separation conditions is very high, typically between 90% and 100%.

Chromatofocusing

Chromatofocusing (CF) is a purification method separating proteins on the basis of differences in their isoelectric points (pI). The matrix is usually a weak anion exchanger in which the functional groups are amines. The eluent is a buffer containing a large number of buffering substances which together give a uniform buffering capacity over a broad pH range. A pH gradient is generated on the column as buffer and medium interacts. Proteins with different pI values migrate at different rates as the pH gradient develops, continually binding and dissociating while being focused into narrow bands and finally eluted.

CF is a powerful method and can resolve very small differences in pI (down to 0.02 pH units) and thus separates very similar proteins. However, the capacity of the method is low and should preferably only be used for partially pure samples. CF can be considered if IEX or other methods do not give satisfactory purification.

Chapter 2
Ion exchange in practice

Introduction

This chapter includes practical advice on how to control experimental conditions to achieve a successful separation and guidelines for selection of the most appropriate medium or prepacked column for each application. The final resolution of an ion exchange (IEX) separation is determined by selectivity and column efficiency. These parameters are influenced in turn by factors such as particle size, porosity and column packing. The separation is influenced by a number of factors, for example, the way in which the net surface charge of each protein in the sample varies with pH, the pH and ionic strength of buffers, and the elution conditions. Understanding the role and importance of each parameter ensures that every separation can be performed with the required resolution, throughput and speed. Additional application examples and product-related information are found in Chapter 3.

Selecting chromatography media

The origin and differences between modern IEX matrices are explained in Chapter 1. Choice of a suitable matrix depends on factors such as the scale of the final purification, the purpose of the separation (for example to concentrate sample in a capture step or to achieve high resolution in a final polishing step) and the throughput required. Refer to Chapter 4 for more details on the use of *capture, intermediate purification, and polishing steps* in a purification strategy.

Capture

When IEX is used as a capture step, the objective is to quickly absorb the protein(s) of interest from the crude sample and isolate them from critical contaminants such as proteases. The target protein(s) are concentrated and transferred to an environment that will conserve potency/activity. Removal of other critical contaminants can also be achieved by careful optimization of pH and elution conditions.

The focus is on capacity and speed in a capture step. It is advisable to compromise on the potential resolution that can be achieved by an IEX separation to maximize the capacity and/or speed of the separation in this first step (Fig 2.1).

Intermediate purification

When IEX is used for intermediate purification, the objective is to remove most of the significant impurities such as proteins, nucleic acids, endotoxins, and viruses. In a typical intermediate purification step, speed is less critical since sample volume has been reduced and damaging contaminants have been removed during capture. Focus is on capacity and resolution in order to maintain productivity and to achieve as high selectivity (purity) as possible (Fig 2.1).

Polishing

When IEX is used for polishing, most impurities have been removed except for trace amounts or closely related substances such as structural variants of the target protein, nucleic acids, viruses, or endotoxins. The purpose of the separation is to reduce these variants and trace contaminants to acceptable levels for the application. In contrast to capture steps where fast, high capacity, step elution is most commonly used, a polishing step will therefore focus on achieving the highest possible resolution (Fig 2.1).

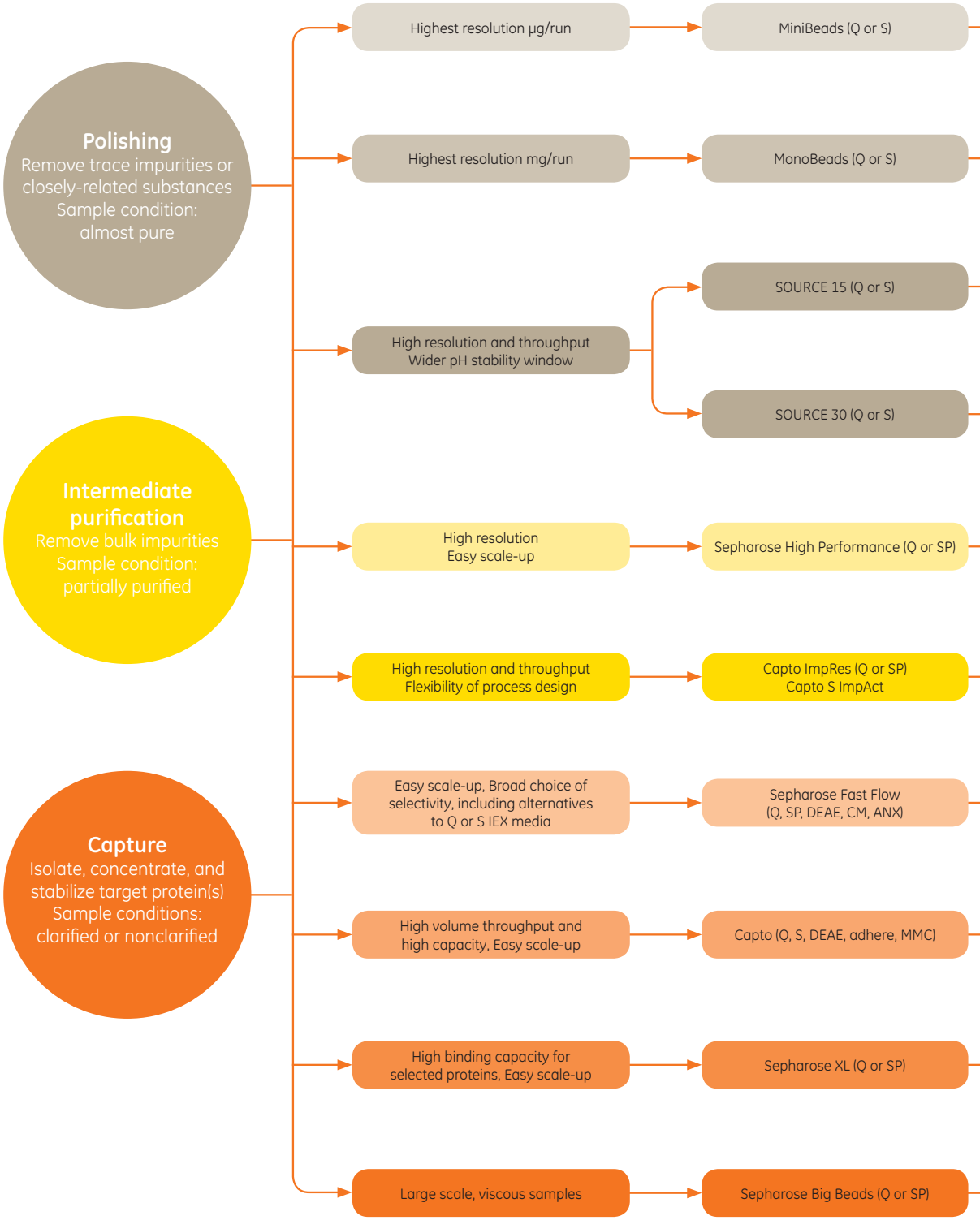
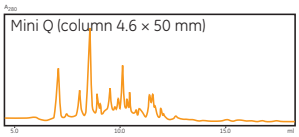


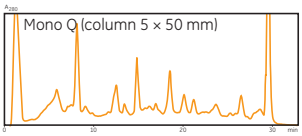
Fig 2.1. A typical purification strategy has three phases: Capture, Intermediate Purification, and Polishing (CIPP). Each phase has a specific objective, dependent largely on the properties of the starting material. The appropriate IEX medium is selected according to the objective of the purification step and the condition of the starting material.

Use for intermediate purification if column capacity is sufficient and no scale-up is required.



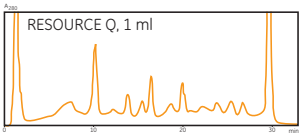
Sample:
Pancreatin
Gradient elution

Use for intermediate purification if column capacity is sufficient and no scale-up is required. Can be used for capture steps if sample is free from particulate matter.



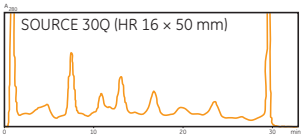
Sample:
Pancreatin
Gradient elution

Use SOURCE 15 when resolution is top priority.



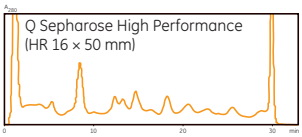
Sample:
Pancreatin
Gradient elution

Use SOURCE 30 when speed is top priority.



Sample:
Pancreatin
Gradient elution

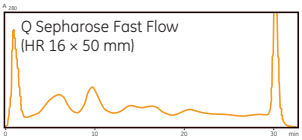
Use HiTrap columns prepacked with Sepharose High Performance, Sepharose XL and Sepharose Fast Flow for media selection and pH scouting.



Sample:
Pancreatin
Gradient elution

Use Capto ImpRes and Capto S ImpAct for increased productivity in large-scale production

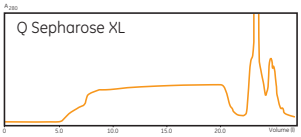
Try weak ion exchangers such as DEAE, CM, or ANX if the selectivity of Q or S is unsatisfactory.



Sample:
Pancreatin
Gradient elution

Use high bed heights for increased productivity. Use Capto MMC for high salt feed.

Use for capture with high binding capacity/rapid separation of proteins from clarified samples



Sample:
Recombinant α-amylase
Pilot scale:
Gradient elution begins after 20 l

Use with step elution.

Resolution

Fast IEX media selection and method development

Fig 2.2. HiTrap™ IEX Selection Kit contains seven HiTrap columns prepacked with different Sepharose Fast Flow media. The kit is an excellent choice for screening of the most appropriate media and conditions to use in application and development work.

Time and sample can be saved in the early stages of development by using small, prepacked columns such as those in the HiTrap IEX Selection Kit (Fig 2.2). The kit allows quick and efficient screening for the most suitable charge group and enables development of the basic separation method (see Appendix 11, *Media selection*). This approach is particularly helpful if the properties of the target protein(s) are unknown.

HiTrap columns can be run with a syringe, a peristaltic pump, or any ÄKTA™ chromatography system. HiTrap columns can be used for small-scale purification as well as fast method development and are supplied with detailed protocols for use.

The HiScreen™ column format has been specially designed for screening and optimizing before scaling up the purification. The higher bed height of HiScreen (10 cm), in comparison with HiTrap (2.5 cm), is suitable for scaling up while keeping the bed height constant.

Practical considerations for IEX separation

This section covers detailed aspects of each step in an IEX separation, together with practical hints and tips to improve resolution and overall performance.

Buffer pH and ionic strength

Buffer pH and ionic strength must be compatible with protein stability and activity. The most suitable pH should allow the proteins of interest to bind, but should be as close to the point of release (elution) as possible. If the pH is too low or too high, elution becomes more difficult and high salt concentrations might be needed. This should be avoided since some proteins begin to precipitate at high ionic strength and high salt concentrations can interfere with assays or subsequent chromatographic steps.

Avoid extreme changes in pH or other conditions that can cause inactivation or even precipitation.

The pH and ionic strength of the sample are extremely important in order to achieve the most effective high resolution or group separations and to make the most of the high loading capacity. Samples should preferably be buffered in the start buffer (see Appendix 1, *Sample preparation* for details). When working with small volumes during screening and scouting, it might be sufficient to dilute the sample in start buffer in order to lower the ionic strength and adjust the pH to a value similar to that of the start buffer.

Proteins often begin to dissociate from IEX media about 0.5 pH units from their pI at an ionic strength around 100 mM. The pH of the start buffer should be at least 0.5 to 1.0 pH unit above the pI of the target substance when using an anion exchanger (Q, DEAE, or ANX) or 0.5 to 1.0 pH unit below the pI of the target substance when using a cation exchanger (SP or CM).

For samples with unknown charge properties, try the following strong ion exchangers first:

anion exchange (Q)
start buffer: pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

cation exchange (S, SP)
start buffer: pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

See Appendix 2 for recommendations on volatile and nonvolatile buffer systems for anion and cation exchangers.

Whenever possible, check for stability at the pH and ionic strength values selected, especially if recovery of biological activity is a priority.

Anion or cation exchanger

For molecules such as nucleic acids, which carry only negatively charged groups, an anion exchanger is the obvious choice. However, since the net charge of molecules such as proteins (carrying positively and negatively charged groups) depends on pH, the choice is based on which type of exchanger and pH give the desired resolution within the constraints of sample stability. For example, Figure 2.3 shows a theoretical protein which has a net positive charge below its pI, and can bind to a cation exchanger. Above its pI, the protein has a net negative charge and can bind to an anion exchanger. However, the protein is only stable in the pH range between 5.0 and 8.0 and so an anion exchanger has to be used.

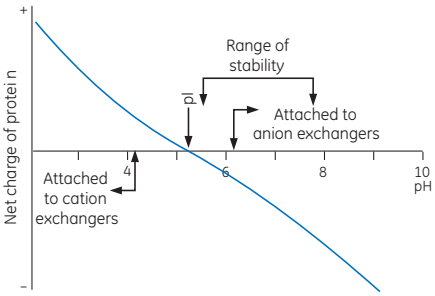


Fig 2.3. Considerations when selecting a suitable IEX medium.

If sample components are most stable below their pI, use a cation exchanger.

If sample components are most stable above their pI, use an anion exchanger.

If stability is high over a wide pH range on both sides of the pI, use either type of ion exchanger.

Strong or weak ion exchangers

Table 2.1 shows the functional groups used on IEX media. The terms strong and weak refer to the *extent that the ionization state of the functional groups varies with pH*. The terms strong and weak do not refer to the strength with which the functional groups bind to proteins.

Table 2.1. Functional groups used on ion exchangers

Anion exchangers		**Functional group**
Quaternary ammonium (Q)	strong	$-CH_2-N^+-(CH_3)_3$
Diethylaminoethyl (DEAE)[1]	weak	$-CH_2-CH_2-N-(CH_2-CH_3)_2$
Diethylaminopropyl (ANX)[1]	weak	$-CH_2-CHOH-CH_2-N-(CH_2^-CH_3)_2$
Cation exchangers		**Functional group**
Sulfopropyl (SP)	strong	$-CH_{2-}CH_{2-}CH_{2-}SO_3^-$
Methyl sulfonate (S)	strong	$-CH_2-SO_3^-$
Carboxymethyl (CM)	weak	$-CH_2-COO^-$

[1] *The active end of the charged group is the same for DEAE and ANX. The difference between them is in the length of the carbon chain of the charged group. DEAE has a diethylaminopropyl group bound to the agarose. ANX has a diethylaminopropyl group attached, which prevents the formation of quaternary groups giving a different selectivity compared to DEAE.*

Begin with a strong ion exchanger to enable development work to be performed over a broad pH range. Use a strong anion exchanger (Q) to bind the protein(s) of interest if their pI is below pH 7.0 or unknown.

Use a strong ion exchanger in cases where maximum resolution occurs at an extreme pH and the proteins of interest are stable at that pH.

Consider using a weak exchanger if the selectivity of the strong ion exchanger is unsatisfactory, but remember that the ion exchange capacity of a weak ion exchanger varies with pH. As a result:

- Sample loading (binding) capacity can vary with increasing pH due to loss of charge from the exchanger.
- Resolution is more readily affected by changes in flow rate or sample load due to the intermediate forms of charge interaction that can occur.
- Predicted results (based on known information about the sample components such as their pI and how their net surface charge changes with pH) might not correlate with actual results since the number of charged groups on weak ion exchangers can vary with pH.
- Longer equilibration times might be required in order to titrate the weak ion exchange functional groups.

When using a weak exchanger, work within the pH values given below to minimize variations in performance:

DEAE: pH 2.0 to 9.0
ANX: pH 2.0 to 9.0
CM: pH 6.0 to 10.0

Buffer selection and preparation

Buffer ions

Buffering ions should have the same charge as the functional groups on the IEX medium (buffering ions that carry a charge opposite to that of the functional groups will take part in the ion exchange process and can cause significant pH fluctuations during elution) and, preferably, a pKa value within 0.6 pH units of the working pH. An exception to this rule is seen in the frequent use of phosphate buffers with anion exchange separations. However, phosphate buffers must be very carefully prepared to ensure reproducibility between batches.

- Use a buffer concentration that is sufficient to maintain buffering capacity and constant pH, typically 20 to 50 mM.
- Use volatile buffers if the purified product is to be lyophilized.

See Appendix 2 for recommendations on volatile and nonvolatile buffer systems for anion and cation exchangers.

- Filter buffers after all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 1 µm filters for particle sizes above 90 µm, 0.45 µm filters for 34 µm particles, or 0.22 µm filters for particles sizes below 15 µm or when sterile or extra clean samples are required. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

Effect of temperature on buffer pH

Select buffers that have appropriate pKa values for the working temperature. The pKa of a buffering substance varies with temperature. For example, Tris has a pKa of 8.85 at 0°C, 8.06 at 25°C and 7.72 at 27°C. Using Tris at 4°C at a pH 7.9 would give a very low buffering capacity and the working pH would be outside the useful pH range (pKa $\pm$ 0.5) of the buffer.

- Prepare buffers at the same temperature at which they will be used.
- Temperatures < 10°C can minimize aggregation caused by hydrophobic interactions between sample components. Working at these lower temperatures can be an alternative solution to using a detergent to improve solubility.

Counterions

- Counterions (salt ions) used in IEX are almost always Na^+ for cation exchange and Cl^- for anion exchange.

Salts such as NaCl have a chaotropic character (i.e., an ability to make water less polar) and therefore a lower 'salting-out' effect on hydrophobic molecules. This ensures maximum solubility during elution and improves recovery. Chaotropic salts can also be used in the presence of organic solvents if required. Salts such as $(NH_4)_2SO_4$ or K_3PO_4 should be avoided as they are most likely to cause precipitation at high concentrations.

In certain applications alternative counterions such as Li^+, Br^-, I^-, SO_4^{2-}, CH_3COO^-, or $HCOO^-$ can improve and even alter, selectivity since they exhibit different elution strengths, but it should be noted that using these ions might affect the binding capacity of the medium. Figure 2.4 shows how selectivity and resolution can vary when using different counterions.

Column: Mono Q HR 5/5
Sample: carbonic anhydrase, transferrin, ovalbumin, α-lactalbumin, β-lactoglobulin A and B

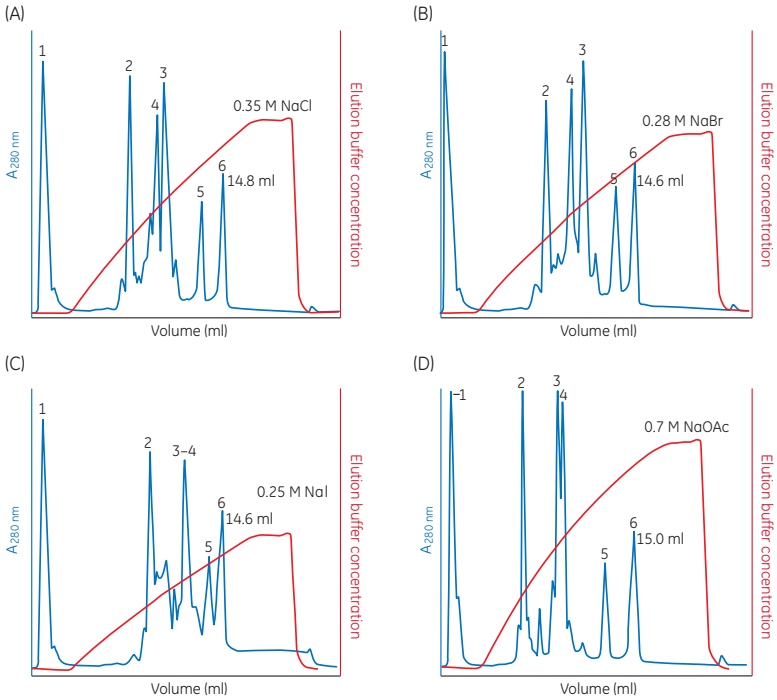


Fig 2.4. Effect of salt ions (counterions) (A) sodium chloride, (B) sodium bromide, (C) sodium iodide, and (D) sodium acetate on selectivity and resolution (Mono Q HR 5/5 now available as Mono Q 5/50 GL). Note the variation in elution order of peaks 3 and 4.

Use the following procedure if the medium is to be used with counterions other than Na^+ or Cl^-:

1. Wash the packed column with 10 CV of 0.5 to 1.0 M salt solution containing the new counterion. Flow rate: see relevant IEX media section in Chapter 3.
2. Wash with 10 CV of start buffer at the same flow rate as in step 1.
3. Repeat steps 1 and 2 several times.

Perform a blank run to check conductivity and pH.

Flow rates

The maximum flow rate applied during a separation can vary according to the stage of the separation. For example, during sample application and elution, lower flow rates allow time for sample components to diffuse in and out of the pores as they to bind to or dissociate from the functional groups. Figure 2.5 shows an example of the influence of flow rate on resolution. Higher flow rates can be used for equilibration, washing and re-equilibration, limited primarily by the rigidity of the media and by pressure specifications of the equipment.

Recommended flow rates for each chromatography medium are given in Chapter 3. Working from these recommendations, *select the highest flow rate that maintains resolution and minimizes separation time.* For example, if peaks are well separated at a low flow rate, increase the flow rate or alternatively, increase the sample volume to benefit from a higher capacity without significant loss of resolution.

Flow rate can be measured as volumetric flow rate, that is, volume per unit time (ml/min). When when comparing results between columns of different sizes or when scaling-up, it is useful to use flow velocity, which measures the flow rate (ml/min) divided by the cross-sectional area of the column and is expressed as flow velocity time (for example, cm/h, see Appendix 5). Results obtained at the same flow velocity on different size columns will be comparable.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

Higher flow rates and viscous buffers increase operating pressures (remember that buffer viscosity increases when running at 4°C). Check the maximum operating pressure of the packed column and set the upper pressure limit on the chromatography system accordingly.

Column:	SOURCE 30Q, 10 mm i.d. × 50 mm (4 ml)
Sample:	Mixture of lactoglobulin B and amyloglucosidase
Sample load:	1 mg/ml of bed volume
Start buffer:	20 mM BIS-Tris PROPANE, pH 7.0
Elution buffer:	500 mM sodium chloride, 20 mM BIS-TRIS PROPANE, pH 7.0
Flow rates (flow velocities):	(A) 4 ml/min (300 cm/h) (B) 13 ml/min (1000 cm/h)
Gradient:	0% to 100% elution buffer, 20 CV

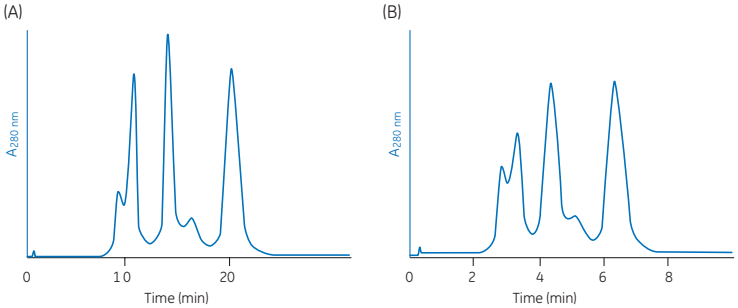


Fig 2.5. Influence of increasing flow rate on resolution.

Flow control

Accurate, reproducible flow control is essential for good resolution and reproducibility.

Use a pump within a chromatography system (rather than a peristaltic pump) to fully utilize the high rigidity and excellent flow properties of the medium.

If you have packed the column yourself, always use a flow rate for separation that is less than the flow rate used for column packing in order to avoid shrinking of the column bed by pressure increases that can occur when running a sample.

Steps in an IEX separation

The six steps listed are described in more detail throughout this section.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable, that is, when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10 to 20 CV with an increasing ionic strength up to 500 mM NaCl (50%B). Alternatively, elute bound proteins with 5 CV of start buffer + NaCl at chosen ionic strength. Repeat at higher ionic strengths until the target protein(s) has been eluted.
5. Regeneration: Wash with 5 CV of 1 M NaCl (100%B) to elute any remaining ionically bound material.
6. Re-equilibrate with 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Buffer volumes referred to are expressed in *column volumes*, for example 3 CV = 3 ml for a column with a 1 ml bed volume. Using CV to describe a separation profile facilitates method development and transfer of methods to columns of different dimensions.

The number of CV used at each stage of the separation can often be reduced by optimization. For example, less buffer is required to equilibrate a strong ion exchanger, the gradient volume can be reduced if resolution can be maintained and less buffer might be required for washing when separating less complex and reasonably clean samples.

Column and media preparation

Using prepacked columns is highly recommended to ensure the desired high performance and reproducible results. An evenly packed column ensures that component peaks are not unnecessarily broadened as sample passes down the column so that optimal resolution can be achieved.

Allow buffers, media or prepacked columns to reach the same temperature before use. Rapid changes in temperature, for example removing packed columns from a cold room and then applying buffer at room temperature, can cause air bubbles in the packing and affect the separation.

Wash away storage solutions and preservatives before using any IEX medium.

Increase the volumes used for column equilibration before the first run if using buffers containing detergents or a different counterion to the one in which the medium has been stored.

Appendix 3 gives details on column packing. The volume required for the packed bed is determined by the amount of sample to be purified and the binding capacity of the medium. Pack a column that will have approximately five-fold excess of the binding capacity required with a bed height up to 20 cm.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3. Note that this does not apply to HiTrap or HiPrep™ columns.

Sample preparation

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Simple steps to clarify a sample before application to a column will avoid the risk of blockage and reduce the need for stringent washing procedures. Appendix 1 contains a detailed overview of sample preparation techniques.

- Desalt samples and transfer into the chosen start buffer (see *Buffer exchange and desalting* in Appendix 1). The pH and ionic strength of the sample are extremely important in order to achieve the most effective high resolution or group separations and to make the most of the high loading capacity.
- For small sample volumes in a high salt concentration and with no major contaminants such as lipids or ionic detergents, it might be sufficient to dilute the sample with start buffer in order to lower the salt concentration to a level that does not interfere with binding to the medium. However, buffer exchange and desalting is the only way to guarantee the correct pH and ionic strength conditions of a sample.
- Samples must be clear and free from particulate matter, particularly when working with particle sizes of 34 µm or less. For small sample volumes, a syringe-tip filter of cellulose acetate or PVDF can be sufficient for sample filtration.

Sample concentration and viscosity

The solubility or viscosity of the sample can limit the quantity that can be applied to a column. High sample viscosity can cause instability of the separation and an irregular flow pattern resulting in broad, distorted peaks, and problems with back pressure. The critical parameter is the viscosity of the sample *relative to* the viscosity of the eluent.

- Dilute viscous samples with start buffer. If high viscosity is caused by the presence of nucleic acid contaminants, see Appendix 1 for advice on their removal. Remember that viscosity varies with temperature. If dilution is not an option, using a medium with a larger particle size can help to overcome viscosity problems.
- Samples should *generally* not exceed 50 to 70 mg/ml protein, but can vary according to the type of sample and the type of chromatographic medium.

Sample application and wash

Starting conditions should maximize binding of the target proteins near the top of the column and, when possible, minimize binding of contaminants so that they pass through the column.

- For efficient binding the sample should be at the same pH and ionic strength as the start buffer. The sample volume can be relatively large without affecting the separation since the sample will bind at the top of the column as long as equilibration and sample conditions are correct.
- Apply samples directly to the column via a chromatography system, a peristaltic pump, or a syringe. The choice of equipment depends largely on the sample volume, the size of column, the type of IEX medium, and the requirements for accuracy in gradient elution. Ensure that the top of the column bed is not disturbed during sample application.
- Do not change buffer conditions until all unbound material has been washed through the column (monitored by UV absorbance) and until UV and conductivity values have returned to starting conditions.

Sample load

Sample load (mass) is of greater importance than sample volume. The amount of sample which can be applied to a column depends on the dynamic binding capacity of the IEX medium and the degree of resolution required. Sample load has a major influence on resolution since the width of the peaks is directly related to the amount of substance present, as shown in Figure 2.6. Consequently, in order to achieve satisfactory resolution, the total amount of protein applied and bound to the medium should not exceed the total binding capacity of the packed column.

Column:	SOURCE 30S, 5 mm i.d. × 50 mm (1 ml)
Sample:	Mixture of chymotypsinogen, cytochrome C, and lysozyme
Sample load:	(A) 1 mg (B) 10 mg
Start buffer:	20 mM sodium phosphate, pH 6.8
Elution buffer:	500 mM sodium chloride, 20 mM sodium phosphate, pH 6.8
Flow rate (flow velocity):	1 ml/min (300 cm/h)
Gradient:	0% to 100% elution buffer, 20 CV

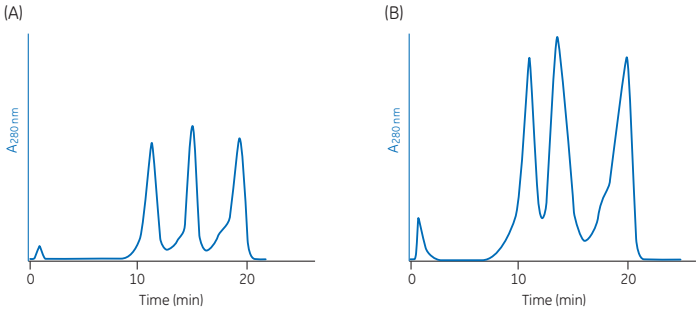


Fig 2.6. The influence of increasing sample load on resolution.

Apply up to 30% of the total binding capacity of the column for optimal resolution with gradient elution. Sample loads can be increased if resolution is satisfactory or when using a step elution.

If sample volumes are large compared to the total CV, the sample buffer composition, in particular the ionic strength, should be the same as that of the start buffer to ensure adequate binding conditions.

Chapter 3 gives typical binding capacities for each medium as a guideline for total binding capacity. The actual (dynamic) binding capacity is also affected by factors such as size and shape of the molecules, the pore size of the matrix, flow rate, sample concentration, pH/protein charge, and ionic strength. Capacity will decrease for molecules of very large diameter or length such as protein complexes > M_r 400 000 asymmetric proteins, and DNA. These molecules are unable to penetrate the matrix pores, limiting their binding primarily to the charged groups on the surface of the matrix. Since the exact distribution of pore sizes in some matrices can vary and the apparent size of a molecule can vary according to the buffer conditions, there is no distinct molecular weight cut-off point when molecules can or cannot penetrate the matrix pores.

The binding step and the dynamic binding capacity can be increased by applying sample at a pH where the target protein has a higher charge than if the optimal pH for separation was used.

Sample volume

As a binding technique, IEX is independent of sample volume as long as the ionic strength of the sample is the same or as low as the start buffer and the target proteins are sufficiently charged at the selected pH. Large volumes of dilute solutions such as fractions from a desalting step or a cell culture supernatant can be applied directly to an IEX medium without prior concentration.

Elution of target protein

Bound proteins are eluted by controlled changes in ionic strength or pH. The way in which these changes take place, by using a linear or step elution, is selected according to the aim of the separation.

Linear gradient elution allows high-resolution separation/analysis. Step elution ensures faster separation time with reduced buffer consumption as well as group separation.

Linear gradient elution

For high-resolution separation/analysis, elution is performed using a linear gradient volume of 10 to 20 CV with an increasing ionic strength up to 500 mM NaCl (50%B).

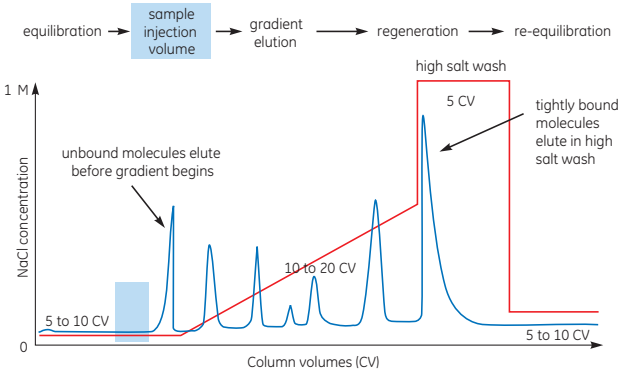


Fig 2.7. Typical IEX separation using linear gradient elution. The UV (protein) and conductivity (salt) traces show the elution of protein peaks and the changes in salt concentration during elution.

Linear ionic strength gradients, as shown in Figure 2.7, are the most frequently used type of elution and should always be used when starting with an unknown sample (when as many components as possible are bound to the column and eluted differentially to see a total protein profile). At low ionic strengths, competition for charged groups on the IEX medium is at a minimum. Increasing the ionic strength increases competition and reduces the interaction between the medium and the bound substances, which begin to elute. The elution buffer is usually the same buffer salt and pH as the start buffer, but contains additional salt, most often sodium chloride.

Use of linear gradient elution during method development is strongly recommended. Linear ionic strength gradients are easy to prepare and very reproducible when generated by a suitable chromatography system. The results obtained can then serve as a base from which to optimize the separation.

The retention of charged proteins on the IEX medium is related to the volume of the column and the concentration difference across it:

- Long, shallow gradients give maximum separation between peaks, but separation times will be longer and there will be greater peak broadening.

- Short, steep gradients give faster separations and sharper peaks, but peaks will be eluted closer together.
- Peaks eluted later in the gradient tend to be slightly broader than those eluted early on.

Select the steepest gradient to give acceptable resolution at the selected pH.

The effects of gradient slope are shown in Figure 2.8.

Column:	Mono Q HR 5/5
Sample:	Partially purified dynorphin-converting enzyme
Start buffer:	20 mM Tris, pH 7.0
Elution buffer:	20 mM Tris, 1 M NaCl, pH 7.0
Flow rate:	1 ml/min

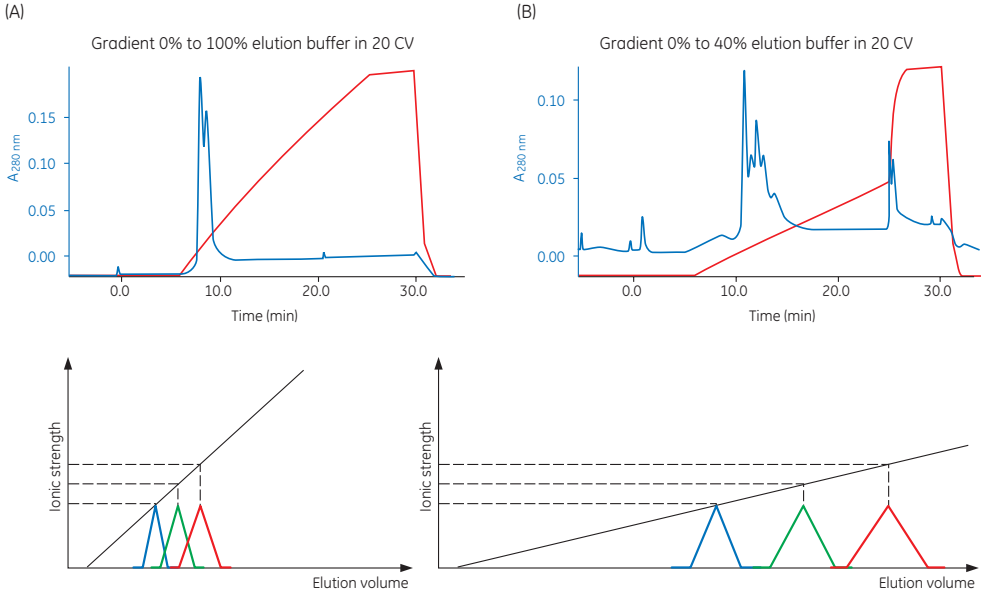


Fig 2.8. Effect of gradient slope on resolution, in theory and in practice.

If gradient elution volumes are decreased, it might be necessary to decrease the sample load proportionally in order to maintain the same resolution. Similarly, if sample load is increased (within the total capacity of the column), gradient volumes might need increasing to maintain resolution.

Gradients are most efficiently formed using ÄKTA chromatography systems with preprogrammed method templates, which automatically control the mixing of solutions being supplied to a column.

Accurate buffer preparation, efficient mixing, and the shortest possible flow path between a mixer and the top of a column will help to ensure accurate gradient formation.

For certain separations, when conditions for a high-resolution separation using a linear gradient have been established, it might be possible to *reduce the total separation time* by using a more complex elution profile while maintaining resolution (Fig 2.9). Shallow gradients can be used where maximum resolution is required while steeper gradients can be used in areas where resolution is satisfactory.

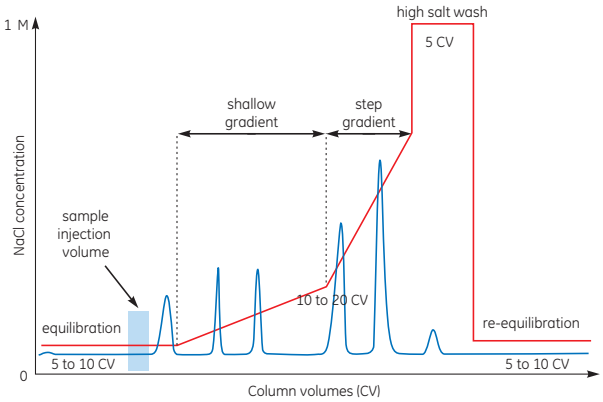


Fig 2.9. Complex gradient profiles can reduce total separation time for certain separations.

Step elution

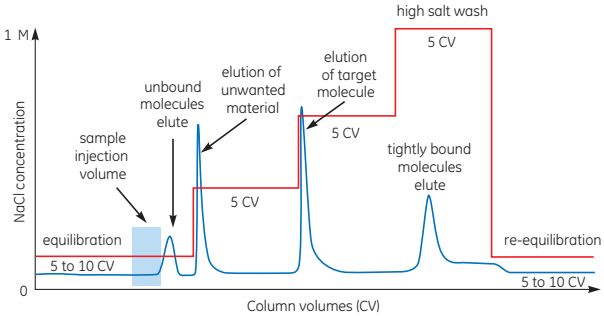


Fig 2.10. Typical IEX separation using step elution. The UV (protein) and conductivity (salt) traces show the elution of protein peaks and the changes in salt concentration during elution.

As shown in Figure 2.10, step elutions are performed by sequential addition of the same buffer at increasing ionic strengths. Step elution is technically simple, but care must be taken in the design of the steps and the interpretation of results since substances eluted by a sharp change in ionic strength elute close together, giving a false peak that can contain several components. Peaks tend to have sharp fronts and pronounced tailing since they frequently contain more than one component. Tailing can lead to the appearance of false peaks if a change in ionic strength is introduced too early. For these reasons, use a linear ionic strength gradient when developing a new method.

When an IEX separation has been optimized using gradient elution, changing to step elution reduces the total number of CV used for a separation. This *speeds up separation times and reduces buffer consumption* while retaining the required purity level. Step elutions of this type are often used for routine, large-scale separation. An added advantage of a step elution when used at larger scale is that it is often possible to apply a greater amount of sample, since the molecules which would elute early in a gradient separation no longer take up binding capacity on the column.

In a *group separation* the molecules of interest are concentrated and rapidly removed from unwanted substances. When binding and elution conditions for a target protein(s) and contaminants have been determined, usually during preliminary gradient elution separations, conditions are chosen to maximize binding of the target protein(s) and minimize binding of contaminants during sample application. The target protein(s) is then eluted by a single buffer change in an enriched, concentrated form. Figure 2.11 shows an example of such a separation in which a HiTrap Q HP column is used to separate human serum proteins from the unwanted IgG fraction, which passes directly through the column.

Column:	HiTrap Q, 1 ml
Sample:	Human serum, filtered (0.45 µm filter) and buffer exchanged to start buffer on a PD-10 column
Sample volume:	1.0 ml
Start buffer:	75 mM Tris-HCl, pH 8.0
Elution buffer:	75 mM Tris-HCl, 1.0 M NaCl, pH 8.0
Flow rate (flow velocity):	0.5 ml/min, (75 cm/h)

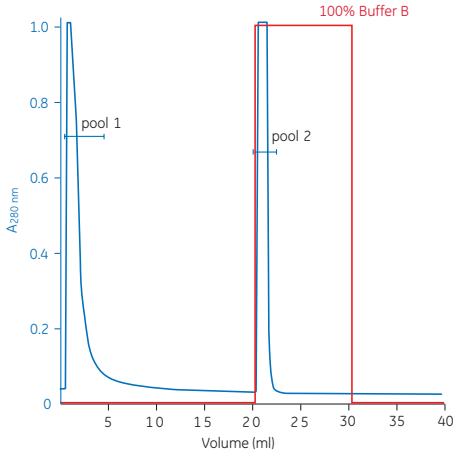


Fig 2.11. Group separation of serum proteins on HiTrap Q HP.

If starting conditions have been chosen to maximize the binding of contaminants, then no change in elution conditions is required since the target protein(s) will pass through the column. For many applications, it is preferable to discard the column rather than spend time and effort removing unwanted bound substances.

pH elution

Since the net charge on a protein is pH-dependent, samples can also be eluted from an IEX medium by altering the pH of the elution buffer. As there is no salt gradient, samples are simply retained on the column at one pH and eluted by increasing or decreasing the pH. The various charged groups in the sample or on the column are titrated until they are neutral or of opposite charge to the medium and the sample elutes.

- Proteins bound to an anion exchanger (Q, DEAE, ANX) will elute as pH is decreased.
- Proteins bound to a cation exchanger (SP, S, CM) will elute as pH is increased.

Since pH elution will involve working at pH values close to the pI of a protein and since many proteins show minimum solubility close to their pI, precautions must be taken to avoid precipitation on the column (see *Detergents, denaturing agents, and other additives* later in this chapter for information on the use of additives to avoid precipitation).

Always test in advance the solubility of sample components at the pH and salt concentrations to be used during separation.

For any type of pH elution, care must be taken in the selection and mixing of buffer systems in order to achieve reproducibility. Stepwise pH elution is easier to produce and more reproducible than using a linear pH gradient. Note that for weak ion exchangers the buffer might have to titrate the charged groups on the medium and there will be a short period of re-equilibration before the new pH is reached.

Linear pH gradients are very difficult to produce at constant ionic strength, since simultaneous changes in ionic strength, although small, also occur. These gradients cannot be obtained simply by mixing buffers of different pH in linear volume ratios since the buffering capacities of the systems produced are pH-dependent. A relatively linear gradient can be produced over a narrow pH interval (maximum 2.0 pH units) by mixing two solutions of the same buffer salt adjusted to 1.0 pH unit above and 1.0 pH unit below the pKa for the buffer.

In general, separation of proteins according to their pI, using chromatofocusing, is likely to provide a more reliable and higher resolution result than attempting to elute proteins from an IEX column using a pH gradient.

Regeneration and re-equilibration

Include a wash step (regeneration) at the end of every run in order to remove any molecules that are still bound to the medium. Monitor UV absorbance so that the wash step can be shortened or prolonged, as necessary.

A re-equilibration step after washing returns the column to start conditions before applying further samples. Whenever possible, monitor pH and conductivity to check when start conditions have been reached. The re-equilibration step can then be shortened or prolonged as necessary.

Increase flow rates during wash and re-equilibration steps to save time between runs.

If ionic detergents have been used, wash the column with 5 CV of distilled water, followed by 2 CV of 2 M NaCl. Re-equilibrate with at least 10 CV of start buffer until the UV baseline, eluent pH, and/or conductivity are stable. Organic solvents such as ethanol can be used to remove nonionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Detergents, denaturing agents, and other additives

Any additives used for dissociation, solubilization, metal chelation, and enzyme inhibition should always be checked for their charge characteristics at the working pH. Run blank gradients with additives included in order to check their effect on the chromatographic profile.

Additives used during sample preparation will be separated from the sample components during IEX. If proteins are seen to precipitate, elute later than expected, or are poorly resolved, add a suitable concentration of the additives used for initial solubilization to the start and elution buffers.

Zwitterionic additives such as betaine can prevent precipitation and can be used at high concentrations without interfering with the gradient elution.

Detergents are useful as solubilizing agents for proteins with low aqueous solubility such as membrane components. Anionic, cationic, zwitterionic, and nonionic (neutral) detergents can be used during IEX.

Denaturing agents such as guanidine hydrochloride or urea can be used for initial solubilization of a sample and during separation. However, they should be avoided unless denaturation is a requirement. Note that, at the pH values used for separation, guanidine is a charged molecule with a counterion and will therefore participate in the ion exchange process in the same way as NaCl.

Examples of commonly used detergents and denaturing agents are given in Table 2.2.

Table 2.2. Commonly used detergents and denaturing agents

Detergent	Type	Typical conditions for use	Compatibility
Urea		2 to 8 M	anion or cation exchangers
Guanidine hydrochloride		3 to 6 M	anion or cation exchangers
Triton™ X-100	nonionic	2%	anion or cation exchangers
N-Octylglucoside	nonionic	2%	anion or cation exchangers
Sodium dodecyl sulfate	ionic	0.1% to 0.5%	exchange for nonionic detergent during first chromatography step, avoid anion exchangers
Sarcosyl[1]	anionic	1.5%	cation exchangers
Nonidet P40	nonionic		anion or cation exchangers
Polyoxyethylene ethers (e.g., Brij 35)	nonionic		anion or cation exchangers
Polyoxyethylene sorbitans (e.g., Tween™ 80)	nonionic		anion or cation exchangers
CHAPS	zwitterionic, derivative of cholic acid		anion or cation exchangers (pH-dependent)
CHAPSO	zwitterionic, derivative of cholic acid		anion or cation exchangers (pH-dependent)
Deoxycholate	cation		anion exchangers

[1] *Sarcosyl is strongly protein-denaturing.*

Temperatures < 10°C can minimize aggregation caused by hydrophobic interactions between sample components. Working at these lower temperatures is an alternative to using a detergent to improve solubility.

Developing or optimizing a separation using buffers that contain detergents

1. Select detergents that are compatible with the sample. A detergent must be neutral, zwitterionic, or have the same charge as the IEX medium. Detergents that bind to the medium can be difficult to remove and can affect protein loading capacity, pH, conductivity, and resolution.
2. Determine the minimum concentration that is likely to keep the sample in solution during the separation. Note that different detergents will have different solubilization properties resulting in different peak profiles.
3. Equilibrate the column thoroughly with the detergent solution, using a concentration that is below the critical micelle concentration for the specific detergent.
4. Run blank salt gradients to determine the UV absorbance profile of the detergent and to detect any effect pH. Micelle formation causes light scattering and the appearance of a peak during UV monitoring. If micelle formation is a problem try the following:
   - decrease detergent concentration as far as possible without impairing sample solubility
   - increase detergent concentration to run the gradient above the critical micelle concentration (this creates a gradual rather than abrupt UV increase)
   - change the salt gradient so that the sudden change in UV absorption does not occur during the run
   - change to highly chaotropic salts such as lithium perchlorate or sodium trichloroacetate that can be used at higher concentrations without causing micelle formation
5. Perform test runs with sample to find the detergent that gives optimal solubilization and resolution.

- A single peak obtained from a 'detergent run' often contains more than one component and should be analyzed carefully. Selecting a different detergent might improve the separation.
- Detergent concentrations that are too high will increase buffer viscosity so that flow rates must be reduced to avoid overpressure of the column. The concentration of detergent required for solubilization can often be reduced during the separation.
- Use detergents of the highest quality that are free from salts. Filter buffers that contain detergents under weak suction and ultrasonication for degassing to avoid foaming.
- Wash previously used columns thoroughly using recommended procedures before working with buffers that contain detergents.

Reagents to reduce polarity

Monoethylene glycol, glycerol and similar mild reagents that reduce polarity can be included in buffers. Avoid high concentrations (> 40% w/w) as buffer viscosity will increase and can overpressure the column.

Metal chelators: EDTA, EGTA

EDTA (ethylenediaminetetracetic acid) and EGTA (ethylene glycol-bis-[2-aminoethyl]-N,N,N',N'-tetraacetic acid) are often used in buffers as metal chelators and can be used with IEX. EDTA and EGTA contain several carboxylic acid groups that interact with anion exchangers. During anion exchange separations, EDTA and EGTA can concentrate as a band on the column and elute during a salt gradient. Both molecules absorb UV and will appear as a peak or as background noise in the chromatogram.

Analysis of results and further steps

The analysis of results from the first separation will indicate if conditions can be improved to increase the yield, achieve higher purity, speed up the separation, or increase the amount of sample that can be processed in a single run.

Samples eluted using a salt gradient will contain a range of salt concentrations. Dilute or desalt fractions before analysis, if the assay is sensitive to changes in salt concentration.

Commonly used analytical assays are outlined in Appendix 8.

Scaling-up

For fast separations, it might be easier to repeat a separation several times on a small column and pool the fractions of interest, rather than scale-up to a larger column. However, a larger column can be preferred for routine processing of large sample volumes. General guidelines for scaling-up are shown in Table 2.3.

Table 2.3. Guidelines for scaling-up

Maintain	Increase
Column bed height	Column volume, i.e., column diameter
Flow velocity (cm/h)	Flow rate (ml/min)
Sample concentration	Sample load
Gradient elution volume , i.e., number of column volumes used for the gradient	

When scaling-up an IEX separation, follow the points below to ensure the same cycle time for small scale and larger scale separations.

1. Optimize the separation at small scale.
2. Maintain bed height, sample concentration, and the ratio of sample volume: volume of medium.
3. Increase the column volume by increasing the cross-sectional area (diameter) of the column.
4. Run the separation at the same flow velocity (see Appendix 5) as used on the smaller column with the same ratio of gradient volume: column volume.

The HiScreen column format has been especially designed for screening and optimizing before scaling up. The higher bed height of HiScreen (10 cm), in comparison with HiTrap (2.5 cm), is suitable for scaling up while keeping the bed height constant.

During method development, a small particle size may be used to improve resolution. However, smaller particles can also result in increased back pressure and this factor can become restrictive when scaling-up. Consider using larger particles, preferably of the same medium, to utilize lower back pressures and higher flow rates.

When scaling-up, the salt concentrations at which peaks elute can decrease with increased sample loads. As sample is applied to the column, components with a low net charge are displaced by components with a higher net charge. Molecules will elute in the same order, but at a different point in the elution profile.

When method scouting, develop the method, whenever possible, on the medium that will be used at the larger scale.

For production-scale separations which must satisfy throughput and cleaning-in-place (CIP) requirements of the bioprocess industry, transfer an optimized method as early as possible to a matrix designed for bioprocessing such as SOURCE, Sepharose High Performance, Sepharose Fast Flow, Capto, or Sepharose Big Beads.

See Appendix 3 for column selection and packing.

Equipment selection

Appendix 4 provides a guide to the selection of systems for IEX.

Care of IEX media

When IEX media have been in use for some time, it might become necessary to remove precipitated proteins or other contaminants that have built up in the column. The need for cleaning can be seen by the appearance of a colored band at top of the column, a space between the upper adapter and the bed surface, a loss in resolution, or a significant increase in back pressure. A general cleaning procedure for each IEX medium is given in Chapter 3 and Appendix 10 also contains recommended procedures for severe contamination by precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins. In all cases, prevention is better than cure and routine cleaning is recommended.

Always use filtered buffers and samples to reduce the need for additional column maintenance. See Appendix 1 for further details on sample preparation.

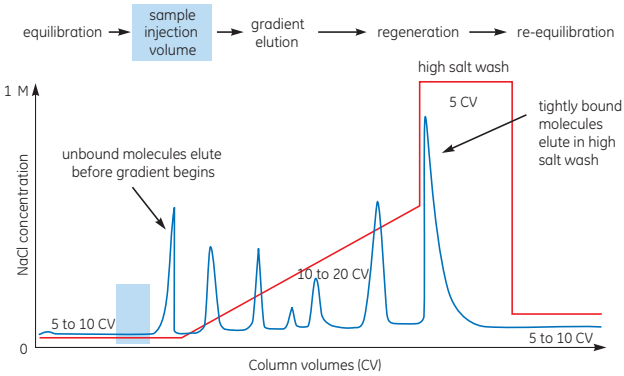
Always degas buffers and keep buffers, columns and samples at the same temperature to avoid the formation of air bubbles in the column.

Filter cleaning solutions before use and always re-equilibrate the column with start buffer before the next separation.

If an increase in back pressure is observed, either on the pressure monitor or by seeing the surface of the medium move downwards, check that the problem is actually caused by the column before starting the cleaning procedure. Disconnect one piece of equipment at a time (starting at the fraction collector), start the pump, and check the pressure after each piece is disconnected. A dirty on-line filter is a common cause of increased back pressure. Check back pressure at the same stage during each run, since the value can vary within a run during sample injection or when changing to a different buffer.

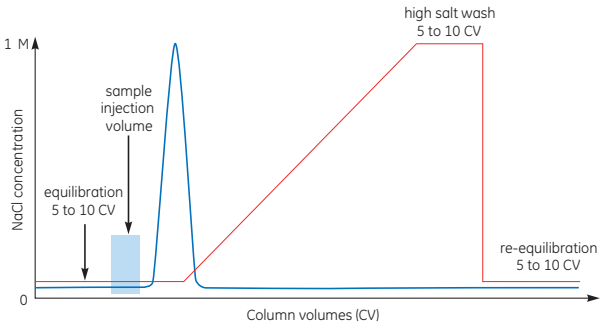
Troubleshooting

The desired IEX separation: target proteins well-resolved by gradient elution



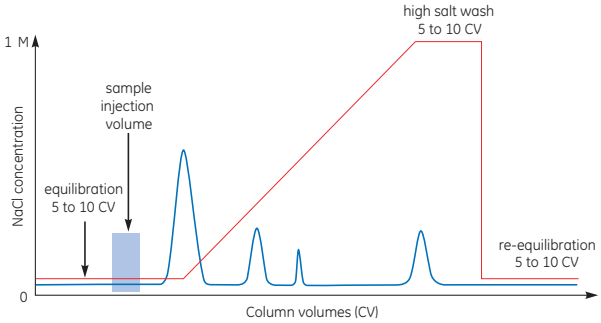
If only certain peaks are of interest in this well-resolved, gradient-elution separation, it can be advantageous to transfer to a step elution in order to save time and buffer. The rest of this section focuses on practical problems that might lead to a suboptimal IEX separation.

Sample elutes before salt gradient begins



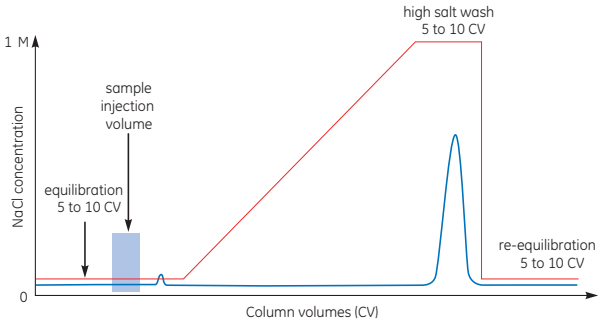
Ensure that buffers are in the correct containers. Reduce ionic strength of sample by desalting (see *Buffer exchange and desalting* in Appendix 1), or dilution with start buffer. For an anion exchanger, increase buffer pH, for a cation exchanger, decrease buffer pH. If proteins still do not bind at any pH, the column might be contaminated by detergent.

Sample still eluting when gradient begins



After sample application the UV trace must return to baseline before elution begins, otherwise proteins that do not bind to the column interfere with the separation. Increase the volume of start buffer (equilibration step) before starting the gradient elution.

Sample elutes during high salt wash



Proteins are binding too strongly. Ensure that buffers are in the correct containers. If using an anion exchanger, decrease buffer pH, if using a cation exchanger, increase buffer pH.

Protein(s) of interest eluting late in gradient

Proteins are binding too strongly. Increase ionic strength of gradient. It is preferable to alter pH if a very high salt concentration is required for elution. For an anion exchanger, decrease buffer pH and for a cation exchanger, increase buffer pH. Refer also to Table 2.4.

Protein(s) of interest eluting too early in gradient

Proteins are not binding strongly. Check ionic strength of gradient. Alter pH, for an anion exchanger, increase buffer pH and for a cation exchanger, decrease buffer pH. Refer also to Table 2.4.

Proteins(s) of interest not sufficiently resolved

Refer to the contents of this chapter to review key parameters for improving resolution.
Refer also to Table 2.4.

Table 2.4. Troubleshooting

Situation	Cause	Remedy
Reduced or no flow through the column	Outlet closed or pumps not working	Open outlet. Check pumps for signs of leakage (if using a peristaltic pump, check tubing also).
	Blocked filter, end-piece, adapter, or tubing.	Remove and clean or replace if possible. Always filter samples before use.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins and aggregates during sample preparation, see Appendix 1. Follow cleaning procedures, see Appendix 10.
	Protein precipitation in the column.	Modify buffer, pH and/or salt conditions during the run to maintain stability. Follow cleaning procedures, see Appendix 10.
	Protein precipitation in the column caused by removal of stabilizing agents during separation.	Modify start buffer and elution buffer to maintain stability.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol[1] to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, see Appendix 10.
Peak of interest is poorly resolved from other major peaks.	Sample applied incorrectly.	Check bed surface and top filter for possible contamination.
	Large mixing spaces at top of or after column.	Adjust top adapter to surface of medium if necessary. Reduce all post-column volumes.
	Incorrect buffer pH and/or ionic strength.	Check pH and ionic strength to ensure that column was re-equilibrated after previous run. Check conditions required. Prepare new solutions.
	Suboptimal elution conditions, e.g., incorrect pH, gradient too steep, flow rate too high.	Alter elution conditions: alter pH, use shallower gradient, reduce flow rate (listed in priority order).
	Sample is too viscous.	Dilute with buffer. Maintain protein concentration below 50 mg/ml.
	Column is poorly packed.	Check column efficiency, see Appendix 3. Repack if needed. Use prepacked columns.
	Column overloaded.	Decrease sample load.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins and aggregates during sample preparation (see Appendix 1).
	Precipitation of proteins in the column.	Modify buffer, pH and/or salt conditions during the run to maintain stability.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol[1] to prevent microbial growth. Always filter buffers. Follow cleaning procedures, see Appendix 10.
Proteins do not bind or elute as expected.	Proteins or lipids have precipitated on the column or column filter.	Clean the column and exchange or clean the filter. Check pH and salt stability of sample.
	Sample not filtered properly.	Clean the column, filter the sample and repeat.
	Sample has changed during storage.	Prepare fresh samples.
	Protein might be unstable or inactive in the elution buffer.	Determine the pH and salt stability of the protein.
	Column equilibration incomplete.	Repeat or prolong the equilibration step until conductivity and pH are constant.

Continues on following page

Table 2.4 cont.

Situation	Cause	Remedy
	Incorrect buffer pH and/or ionic strength.	Check conditions required. Prepare new solutions.
	Proteins are forming aggregates and binding strongly to the medium.	Use urea or zwitterions, betaine up to 10%, taurine up to 4%.
	Sample or buffer conditions are different from previous runs.	Check sample and buffer conditions.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, see Appendix 10.
Protein elutes later than expected or not at all.	Incorrect buffer pH.	Check pH meter calibration. Use a buffer pH closer to the pI of the protein.
	Ionic strength too low.	Increase salt concentration in elution buffer.
	Ionic interactions between protein and matrix.	Maintain ionic strength of buffers above 50 mM.
	Hydrophobic interactions between protein and matrix.	Reduce salt concentration to minimize hydrophobic interaction. Increase pH. Add suitable detergent or organic solvent, e.g., 5% isopropanol.
Protein elutes earlier than expected (during the wash phase)	Ionic strength of sample or buffer is too high.	Decrease ionic strength of sample or buffer.
	Incorrect pH conditions.	Increase pH (anion exchanger). Decrease pH (cation exchanger).
	Column equilibration incomplete.	Repeat or prolong the equilibration step until conductivity and pH are constant.
Leading or very rounded peaks in chromatogram.	Channeling in the column.	Repack column using a thinner slurry of medium. Check column packing (see Appendix 3).
	Column overloaded.	Decrease sample load and repeat.
	Column contaminated.	Clean using recommended procedures.
Peaks are tailing.	Incorrect start buffer conditions, sample is not binding to column.	Adjust pH. Check salt concentration in start buffer.
	Sample too viscous.	Dilute in application buffer.
	Column packing too loose.	Check column efficiency (see Appendix 3). Repack using a higher flow rate. Use prepacked columns.
Peaks have a leading edge.	Column packing compressed.	Check column efficiency (see Appendix 3). Repack using a lower flow rate. Use prepacked columns.
Medium/beads appears in eluent.	Column packing compressed.	Check column efficiency (see Appendix 3). Repack using a slower flow rate. Use prepacked columns.
	Bed support end piece is loose or broken.	Replace or tighten.
	Column operated at too high pressure.	Do not exceed recommended operating pressure for medium or column.
	Medium has been damaged during column packing.	Do not use magnetic stirrers when equilibrating loose IEX medium
Low recovery of activity, but normal recovery of protein.	Protein might be unstable or inactive in the buffer.	Determine the pH and salt stability of the protein.
	Enzyme separated from cofactor or similar.	Test by pooling aliquots from the fractions and repeating the assay.

Continues on following page

Table 2.4 cont.

Situation	Cause	Remedy
Protein yield lower than expected.	Protein might have been degraded by proteases.	Add protease inhibitors to the sample and buffers to prevent proteolytic digestion. Run sample through a medium such as Benzamidine Sepharose 4 Fast Flow (high sub) to remove trypsin-like serine proteases.
	Adsorption to filter during sample preparation.	Use another type of filter.
	Sample precipitates.	Check pH and salt conditions, adjust to improve sample solubility.
	Hydrophobic proteins.	Add denaturing agents, polarity reducing agents or detergents. Add 10% ethylene glycol to running buffer to prevent hydrophobic interactions.
	Nonspecific adsorption.	Reduce salt concentration to minimize hydrophobic interaction. Add suitable detergent or organic solvent, e.g., 5% isopropanol[1]. If necessary, add 10% ethylene glycol to running buffer to prevent hydrophobic interactions.
Peaks too small.	Sample absorbs poorly at chosen wavelength.	If appropriate, check absorbance range on monitor. If satisfactory, use a different wavelength, e.g., 214 nm instead of 280 nm.
	Different assay conditions have been used before and after the chromatographic step.	Use same assay conditions for all assays.
	Excessive band broadening.	Check column packing. Repack if necessary.
More sample is recovered than expected.	Protein co-eluting with other substances.	Optimize conditions to improve resolution. Check buffer conditions used for assay before and after the run. Check selection of medium.
More activity is recovered than was applied to the column.	Different assay conditions have been used before and after the chromatography step.	Use same assay conditions for all assays.
	Removal of inhibitors during separation.	
Back pressure increases during a run or during successive runs.	Bed compressed.	If possible repack the column or use a new column. Check sample preparation.
	Microbial growth.	Store in the presence of 20% ethanol to prevent microbial growth. Always filter buffers. Follow cleaning procedures, see Appendix 10.
	Turbid sample.	Improve sample preparation (see Appendix 1). Improve sample solubility: add betaine (max. 10% w/v at 25°C), taurine (max. 4% w/v at 25°C, below pH 8.5) or glycerol (1% to 2 %). For hydrophobic samples, add ethylene glycol, urea, detergents, or organic solvents.
	Precipitation of protein in the column filter and/or at the top of the bed.	Clean using recommended methods. If possible, exchange or clean filter or use a new column. Include any additives that were used for initial sample solubilization in the running buffer.
	Incorrect pH is causing precipitation.	Calibrate pH meter, prepare new solutions and try again. Change pH.
	Precipitation of lipoproteins at increased ionic strength.	Lipoproteins are removed prior to chromatography by the addition of 10% dextran sulfate (final 0.2%), and 1 M calcium chloride (final 0.5 M).

Continues on following page

Table 2.4 cont.

Situation	Cause	Remedy
Air bubbles in the bed.	Buffers not properly degassed.	Degas buffers thoroughly.
	Column packed or stored at cool temperature and then warmed up.	Remove small bubbles by passing degassed buffer through the column; take special care if buffers are used after storage in a fridge or cold room. Do not allow column to warm in sunlight or heating system. Repack column if possible (see Appendix 3).
Cracks in the bed.	Large air leak in column.	Check all connections for leaks. Repack the column if possible (see Appendix 3).
Negative peaks at solvent front.	Refractive index effects.	Exchange the sample into start buffer.
Unexpected peaks in chromatogram.	Buffer impurities.	Clean the buffer by running it through a precolumn. Use high quality reagents.
Peaks appear on gradients.	Incomplete elution of previous sample.	Wash the column according to recommended blank methods.
Spikes in chromatogram.	Air bubble trapped in UV monitor flow cell.	Always use degassed buffers.
UV baseline rises with gradient.	Micelle formation as salt concentration changes.	Work below or above the critical micelle concentration of any detergents being used or change the gradient so that the increase in UV absorption does not occur while the samples are eluting.
	Buffer impurities.	Use high quality reagents.

Chapter 3
Ion exchange chromatography media

Introduction

Historically, several different types of material have been used as a base matrix to which positively or negatively charged groups are covalently attached to form an IEX medium. Chapter 1 describes how the matrix characteristics determine chromatographic properties such as efficiency, capacity, and recovery as well as chemical and physical stability and flow properties.

IEX media such as Sepharose High Performance, Sepharose 6 Fast Flow, SOURCE, and Capto have much improved flow properties compared to earlier media and show no change in bed volume under conditions of changing ionic strength or pH. Stringent conditions can be used for cleaning the media when required and there is no need for frequent column repacking. Most of these media are also designed to meet the throughput and cleaning-in-place requirements for large-scale industrial chromatography. The following descriptions of different IEX media will start from high-resolution media for purification and analysis in small scale, continue with media for many standard applications, and end up with media for larger scales.

MiniBeads: purification or analysis of microgram to milligram quantities with high resolution

- Use MiniBeads for polishing steps at microscale when high resolution is essential and the capacity of the prepacked column is sufficient.
- Use MiniBeads for intermediate purification if only microgram to milligram quantities are required, if there is no requirement for scale-up, and if the capacity of the prepacked column is sufficient. To avoid column blockage, it is especially important to remove particulate matter before using MiniBeads.
- Use MiniBeads for faster, higher resolution separations compared to MonoBeads, if the capacity of the prepacked column is sufficient.
- Run MiniBeads on systems ÄKTA chromatography systems such as ÄKTA pure 25 and HPLC systems. HPLC systems are recommended for optimal performance of the smallest columns (PC 3.2/3). Appendix 4 provides guidance on selecting the right ÄKTA system.

MiniBeads are based on a nonporous, monodispersed matrix of rigid, hydrophilic polymer particles, substituted with quaternary amino (Q) or methyl sulfonate (S) groups. The very small size (3 µm), uniformity and physical rigidity of the particles create excellent conditions for extremely high-resolution ion exchange separations at relatively high flow rates and low back pressures (nonuniform, porous particles would create higher back pressures, reduce flow rate and impair achievable resolution). Such high resolution is essential for successful separation of complex samples in the picogram (pg) to microgram (µg) scale. The strong ion exchange groups (Q and S) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Media characteristics

Composition: rigid, nonporous matrix of monodisperse, hydrophilic polymer particles (3 µm) substituted with quaternary amino (Q) or methyl sulfonate (S) groups (Table 3.1).

Table 3.1. Characteristics of MiniBeads media

Product	Functional group	pH stability[1]	Mean particle size
Strong anion exchanger			
Mini Q	$-CH_2-N^+-(CH_3)_3$	Long term: 3 to 11 Short term: 1 to 14	3 µm (monosized)
Strong cation exchanger			
Mini S	$-CH_2-SO_3^-$	Long term: 3 to 11 Short term: 1 to 14	3 µm (monosized)

[1] *Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance. Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. All ranges are estimates based on experience and knowledge gained at GE.*

Purification options

Mini Q and Mini S™ media are available prepacked in Precision (PC 3.2/3) and Tricorn™ (4.6/50 PE) columns for high-resolution purification of biomolecules (Fig 3.1). The purification options for the prepacked columns are shown in Table 3.2.

Fig 3.1. Mini Q and Mini S media are available prepacked in Precision (PC 3.2/3) and Tricorn (4.6/50 PE) columns.

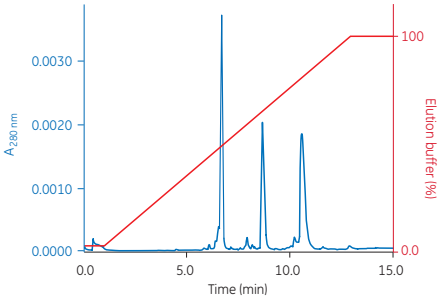
Table 3.2. Purification options for Mini Q and Mini S prepacked columns

Product, column volume	Binding capacity per column	Maximum flow rate (ml/min)	Recommended working flow rate range (ml/min)	Working pH range[1]	Maximum operating back pressure[2] (MPa/psi) *1 MPa = 10 bar*
Strong anion exchangers					
Mini Q PC 3.2/3, 0.24 ml[3]	1.44 mg (α-amylase, M_r 49 000) 1.44 mg (trypsin inhibitor, M_r 20 100)	1.0	0.1 to 1.0	3 to 11	10/1450
Mini Q 4.6/50 PE, 0.8 ml	4.8 mg (α-amylase, M_r 49 000) 4.8 mg (trypsin inhibitor, M_r 20 100)	2.0	0.5 to 2.0	3 to 11	18/2600
Strong cation exchangers					
Mini S PC 3.2/3, 0.24 ml[3]	1.2 mg (ribonuclease, M_r 13 700) 1.2 mg (lysozyme, M_r 14 300)	1.0	0.1 to 1.0	3 to 11	10/1450
Mini S 4.6/50 PE, 0.8 ml	4 mg (ribonuclease, M_r 13 700) 4 mg (lysozyme, M_r 14 300)	2.0	0.5 to 2.0	3 to 11	18/2600

[1] *Working pH range refers to the pH interval where the medium can be operated as intended without adverse long-term effects.*
[2] *Maximum operating back pressure refers to the pressure above which the medium begins to compress.*
[3] *Requires a Precision Column Holder for attachment to HPLC systems, see Appendix 4.*

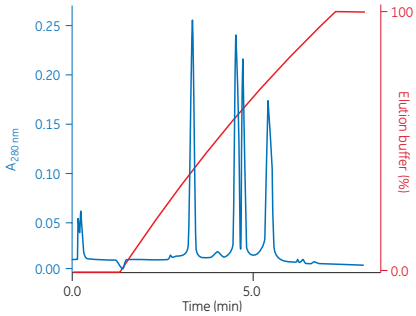
Purification examples

Fast separations at high resolution



Column:	Mini S 4.6/50 PE
Sample:	α-chymotrypsinogen A (25 µg/ml) ribonuclease A (75 µg/ml) lysozyme (25 µg/ml)
Sample volume:	200 µl
Start buffer:	20 mM sodium acetate, pH 5.0
Elution buffer:	20 mM sodium acetate, 400 mM NaCl, pH 5.0
Flow rate:	0.83 ml/min
Gradient:	0% to 100% elution buffer in 12 CV

Fig 3.2. Separation of a protein mixture on Mini S 4.6/50.



Column:	Mini S PC 3.2/3
Sample:	α-chymotrypsinogen A, ribonuclease A, cytochrome C, lysozyme (6:10:6:5), 25 µg/ml
Sample load:	6 µg
Start buffer:	20 mM acetic acid, pH 5.0
Elution buffer:	20 mM acetic acid, 500 mM lithium chloride, pH 5.0
Flow rate (velocity):	0.80 ml/min (10 cm/min)
Gradient:	0% to 100% elution buffer in 6 min (20 CV)

Fig 3.3. Mini S PC 3.2/3 gives fast, high-resolution separation.

Purity check

Column:	Mini Q 4.6/50 PE
Start buffer:	10 mM NaOH
Elution buffer:	10 mM NaOH, 2 M NaCl
Flow rate:	1.0 ml/min

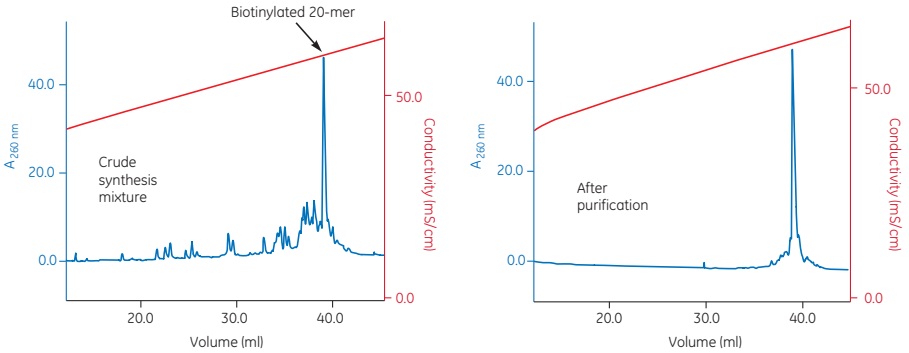
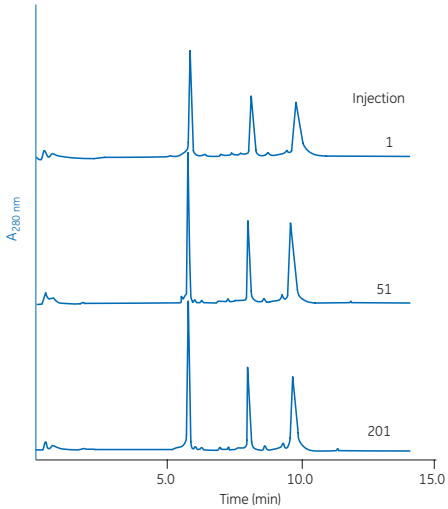


Fig 3.4. Purity check of 5'-biotinylated synthetic oligonucleotide 20-mer on Mini Q 4.6/50 PE before and after purification on a RESOURCE RPC column.

Long-term reproducibility



Column:	Mini S PC 3.2/3
Sample:	Chymotrypsinogen A, ribonuclease A, lysozyme, 6 mg in ratio 1:3:1
Start buffer:	20 mM acetic acid, pH 5.0
Elution buffer:	20 mM acetic acid, 400 mM NaCl, pH 5.0
Flow rate:	0.4 ml/min
Gradient:	0% to 100% elution buffer in 12 min (20 CV)

Fig 3.5. Chromatograms from the first, 51st, and 201st separation of a series run on the same Mini S PC 3.2/3 column. The same consistent reproducibility has been confirmed on Mini Q PC 3.2/3 (data not shown).

Performing a separation

Guidelines for selection of media, buffer, pH, and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance, especially when using small particles such as MiniBeads. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 0.22 µm filters. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

The pH of the start buffer should be at least 0.5 to 1.0 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5 to 1.0 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and nonvolatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

anion exchange (Q)

start buffer: 20 mM Tris-HCl, pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

cation exchange (S)

start buffer: 20 mM 2-(N-Morpholino)ethanesulfonic acid (MES), pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTA systems with automatic buffer preparation functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8.0 or cation exchange chromatography at pH 6.0, see *ÄKTA Laboratory-scale Chromatography Systems: Instrument Management Handbook*, 29010831.

First-time use or after long-term storage

1. To remove ethanol, wash with 4 CV of distilled water at 0.1 ml/min (Mini Q and S PC 3.2/3, 0.24 ml columns) or 0.5 ml/min (Mini Q and S 4.6/50 PE, 0.8 ml columns). This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 4 CV of start buffer at 0.4 ml/min (Mini Q and S PC 3.2/3, 0.24 ml columns) or 0.8 ml/min (Mini Q and S 4.6/50 PE, 0.8 ml columns).
3. Wash with 4 CV of elution buffer, same flow as step 2.
4. Wash with 4 CV of start buffer, same flow as step 2.

Separation by gradient elution

Flow rates: 0.4 ml/min (PC 3.2/3, 0.24 ml columns) or 0.8 ml/min (4.6/50 PE, 0.8 ml columns). Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable, that is, when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10 to 20 CV and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 CV of 1 M NaCl (100%B) to elute any remaining ionically bound material.
6. Re-equilibrate with 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Although separations by step elution (see Chapter 2) can be performed using MiniBeads, gradient elution is recommended to maximize resolution.

If ionic detergents have been used, wash the column with 5 CV of distilled water, followed by 2 CV of 2 M NaCl. Re-equilibrate with at least 10 CV of start buffer until the UV baseline, eluent pH, and/or conductivity are stable. Organic solvents such as ethanol can be used to remove nonionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Refer to Chapter 2 for advice on optimizing the separation. Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

Reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step can vary according to the degree of contamination.

Removing common contaminants

1. Wash with 2 CV of 2 M NaCl at 0.2 ml/min.
2. Wash with 4 CV of 1 M NaOH at 0.2 ml/min.
3. Wash with 2 CV of 2 M NaCl at 0.2 ml/min.
4. Rinse with at least 2 CV of distilled water at 0.2 ml/min until the UV-baseline and eluent pH are stable.
5. Wash with at least 4 CV of start buffer or storage buffer at 0.2 ml/min until pH and conductivity values have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins, refer to Appendix 1.

Chemical stability

For daily use, MiniBeads are stable in all common aqueous buffers in the pH range of 3 to 11 and in the presence of additives such as denaturing agents (8 M urea or 6 M guanidine hydrochloride), nonionic or ionic detergents, and up to 30% acetonitrile in aqueous buffers. Note that aqueous solutions of urea, ethylene glycol, and similar compounds will increase the back pressure due to increased viscosity.

MiniBeads can be used with organic solutions such as dimethylsulfoxide, dimethylformamide, or formic acid, but the separation properties of the media will change.

Avoid anionic detergents with Mini Q. Avoid cationic detergents with Mini S. Avoid oxidizing agents.

Storage

For column storage, wash with 4 CV of distilled water followed by 4 CV of 20% ethanol. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid overpressuring the column. Store at room temperature or, for long periods, store at 4°C to 8°C. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Ensure that the column is sealed well to avoid drying out. Do not freeze.

MonoBeads: purification of milligram quantities with high resolution

- Use MonoBeads for polishing steps at laboratory scale when high resolution is essential and a higher capacity than MiniBeads is required.
- Use MonoBeads for capture or intermediate purification when milligram quantities are required, when there is no requirement for scale-up, and/or when prepacked MiniBead columns do not offer sufficient capacity. Note that, to avoid column blockage, it is especially important to remove particulate matter before using MonoBeads.
- Run MonoBeads on ÄKTA chromatography systems and HPLC systems. Appendix 4 provides guidance on selecting the right ÄKTA system. HPLC systems are recommended for optimal performance of the smallest columns (PC 1.6/5).

Mono Q and Mono S™ IEX media are based on a hydrophilic matrix made from monodispersed, rigid, polystyrene/divinyl benzene particles, substituted with quaternary ammonium (Q) or methyl sulfonate (S) groups (Fig 3.6). This combination confers extreme chemical and physical stability to the media. The small particle sizes (10 µm) allow fast binding and dissociation to facilitate high resolution while the uniformity of the particles ensures high flow rates at low back pressures. The strong ion exchange groups (Q and S) maintain their charge over a broad pH range (Fig 3.7), allowing selection of the most suitable pH for each application.

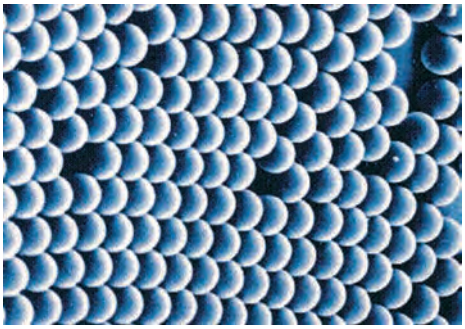


Fig 3.6. Electron micrograph of MonoBeads showing their distinct monodispersity.

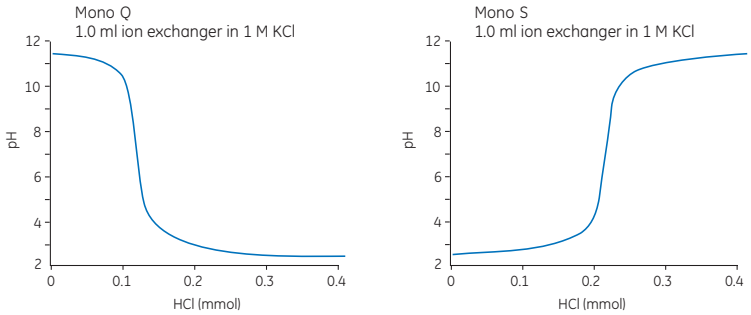


Fig 3.7. Titration curves for Mono Q and Mono S. Binding capacity remains constant over a broad pH working range.

Media characteristics

Composition: rigid, monodisperse, polystyrene/divinyl benzene particles (10 µm) with an optimized pore size distribution. The base matrix is substituted with quaternary amino (Q) or methyl sulfonate groups (S), see Table 3.3.

Table 3.3. Characteristics of MonoBeads media

Product	Functional group	pH stability[1]	Mean particle size (µm)
Strong anion exchanger			
Mono Q	$-CH_2-N^+-(CH_3)_3$	Long term: 2 to 12 Short term: 2 to 12	10 (monosized)
Strong cation exchanger			
Mono S	$-CH_2-SO_3^-$	Long term: 2 to 12 Short term: 2 to 14	10 (monosized)

[1] *Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on chromatographic performance. Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. All ranges are estimates based on experience and knowledge gained at GE.*

Purification options

MonoBeads (Q and S) are available in convenient prepacked Tricorn PE (PEEK) and Tricorn GL (glass) columns (Fig 3.8). Purification options for the prepacked columns are described in Table 3.4.

Fig 3.8. MonoBeads (Q and S) are available prepacked in Tricorn PC (Precision Column), PE (PEEK), and Tricorn GL (glass) columns.

Table 3.4. Purification options for Mono Q and Mono S prepacked columns

Product, column volume	Binding capacity per column	Recommended working flow rate range (ml/min)	Maximum flow rate (ml/min)	Working pH range[1]	Maximum operating back pressure[2] (MPa/psi) *1 MPa = 10 bar*
Strong anion exchangers					
Mono Q PC 1.6/5, 0.1 ml[3]	2.5 mg (thyroglobulin, M_r 669 000)	0.01 to 0.4	0.4	2 to 12	5/725
Mono Q 5/50 GL, 1 ml	25 mg (thyroglobulin, M_r 669 000) 65 mg (HSA, M_r 68 000) 80 mg (α-lactalbumin, M_r 14 300)	0.5 to 3.0	3.0	2 to 12	4/580
Mono Q 4.6/100 PE, 1.7 ml	40 mg (thyroglobulin, M_r 669 000) 110 mg (HSA, M_r 68 000) 140 mg (α-lactalbumin, M_r 14 300)	0.5 to 3.0	3.0	2 to 12	4/580
Mono Q 10/100 GL, 8 ml	200 mg (thyroglobulin, M_r 669 000) 520 mg (HSA, M_r 68 000) 640 mg (α-lactalbumin, M_r 14 300)	2.0 to 6.0	10.0	2 to 12	4/580
Mono Q HR 16/10, 20 ml	500 mg (thyroglobulin, M_r 669 000) 1300 mg (HSA, M_r 68 000) 1600 mg (α-lactalbumin, M_r 14 300)	up to 10.0	10.0	2 to 12	3/435
Strong cation exchangers					
Mono S PC 1.6/5, 0.1 ml[3]	7.5 mg (human IgG, M_r 160 000)	0.01 to 0.4	0.4	2 to 12	5/725
Mono S 5/50 GL, 1 ml	75 mg (human IgG, M_r 160 000) 75 mg (ribonuclease, M_r 13 700)	0.5 to 3.0	3.0	2 to 12	4/580
Mono S 4.6/100 PE, 1.7 ml	130 mg (human IgG, M_r 160 000) 130 mg (ribonuclease, M_r 13 700)	0.5 to 3.0	3.0	2 to 12	4/580
Mono S 10/100 GL, 8 ml	600 mg (human IgG, M_r 160 000) 600 mg (ribonuclease, M_r 13 700)	2.0 to 6.0	10.0	2 to 12	4/580
Mono S HR 16/10, 20 ml	1500 mg (human IgG, M_r 160 000) 1500 mg (ribonuclease, M_r 13 700)	up to 10.0	10.0	2 to 12	3/435

[1] *Working pH range refers to the pH interval where the medium can be operated as intended without adverse long-term effects.*

[2] *Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

[3] *Requires a Precision Column Holder for attachment to HPLC systems.*

Purification examples

Two-step purification using complementary selectivities

Column: Mono Q HR 5/5
Sample: 500 ml of *T. reesei* crude cellulases in start buffer, 2.5 mg
Start buffer: 20 mM Tris-HCl, pH 7.6
Elution buffer: 20 mM Tris-HCl, 500 mM NaCl, pH 7.6
Flow rate: 1.0 ml/min
Gradient: 0% elution buffer (4 CV), 0% to 40% elution buffer (21 CV), 40% to 100% elution buffer (15 CV)

Column: Mono S HR 5/5
Sample: Peak 3 from Mono Q HR 5/5
Start buffer: 20 mM acetate, pH 3.6
Elution buffer: 20 mM acetate, 200 mM NaCl, pH 3.6
Flow rate: 1.0 ml/min
Gradient: 0% to 100% elution buffer (26 CV)

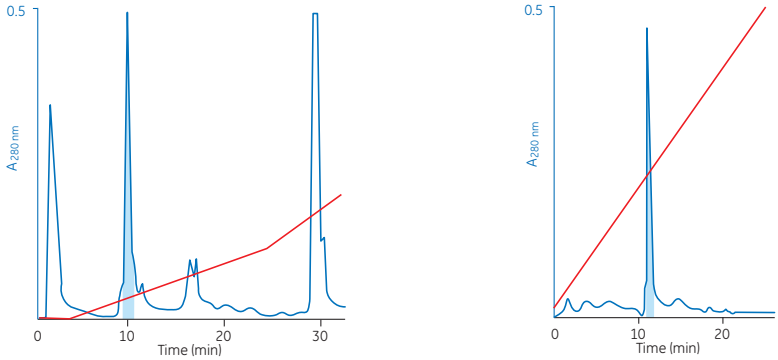


Fig 3.9. Purification of cellulose on Mono Q and Mono S HR 5/5 columns (now available as Mono Q 5/50 GL and Mono S 5/50 GL).

High-resolution polishing step

Column: Mono S 5/50 GL
Sample: Recombinant transposase TniA partially purified on SOURCE 15Q 4.6/100 PE and HiTrap Heparin HP, 5 ml
Sample load: 14.5 ml
Start buffer: 20 mM MES, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, pH 6.5
Elution buffer: 20 mM MES, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 1 M NaCl, pH 6.5
Flow rate: 1 ml/min
Gradient: 0% to 100% elution buffer in 20 CV

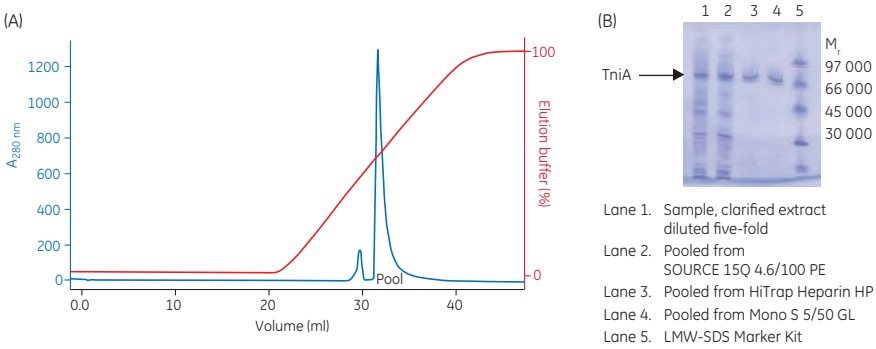
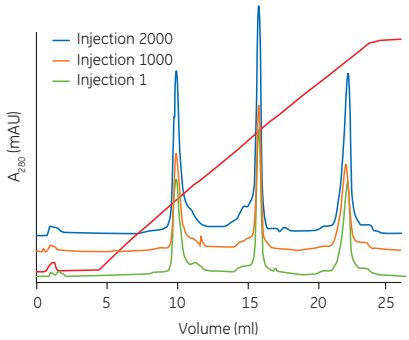


Fig 3.10. Final polishing step in purification of a DNA-binding protein, transposase TniA. Two well-resolved peaks after separation on Mono S 5/50 GL. (A) SDS-PAGE analysis shows fractions from each of the three steps used in this protocol. (B) PhastSystem™ electrophoresis unit using SDS-PAGE PhastGel™ Homogeneous – 12.5 and Coomassie™ staining.

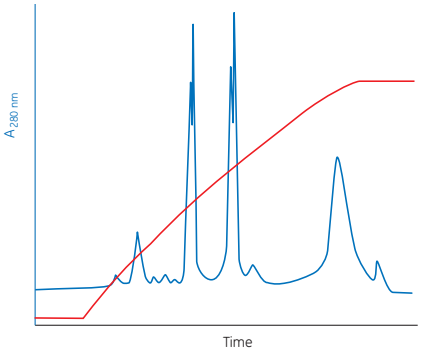
Long-term reproducibility



Column:	Mono Q 5/50 GL (Tricorn)
Sample:	Conalbumin (3.0 mg/ml), α-lactalbumin (4 mg/ml), STI (6 mg/ml)
Sample volume:	200 µl
Buffer A:	200 mM Tris, pH 7.0
Buffer B:	Buffer A + 500 mM NaCl
Gradient:	Linear, 0% to 100% B in 20 CV
Column equilibration:	5 CV
Flow rate:	*1.0 ml/min*
System:	*ÄKTA system*

Fig 3.11. Chromatograms illustrating run to run reproducibility for Mono Q 5/50 GL (Tricorn column). Runs 1, 1000, and 2000 are shown.

Separation in organic solvents



Column:	Mono S HR 5/5
Sample:	Bacitracin 4 mg/ml in start buffer
Sample load:	200 µl
Start buffer:	90% methanol, 50 mM formic acid/lithium hydroxide (LiOH), pH 3.8
Elution buffer:	90% methanol, 50 mM formic acid/LiOH, 350 mM lithium perchlorate ($LiClO_4$), pH 3.8
Flow rate:	1 ml/min
Gradient:	0% to 100% elution buffer in 20 CV

Fig 3.12. Separation of the peptide bacitracin on Mono S HR 5/5 (now available as Mono S 5/50 GL).

Performing a separation

Guidelines for selection of media, buffer, pH, and ionic strength conditions and method optimization are given in Chapter 2. Use these instructions as a basis from which to optimize a separation.

☞ Correct sample and buffer preparation is essential to achieve optimal separation and to avoid any deterioration in column performance, especially when using small particles such as MonoBeads. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

☞ Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 0.22 µm filters. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

☞ The pH of the start buffer should be at least 0.5 to 1.0 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5 to 1.0 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and nonvolatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

anion exchange (Q)

start buffer: 20 mM Tris-HCl, pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

cation exchange (S)

start buffer: 20 mM 2-(N-Morpholino)ethanesulfonic acid (MES), pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTA systems with automatic buffer preparation functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8.0 or cation exchange chromatography at pH 6.0, see *ÄKTA Laboratory-scale Chromatography Systems: Instrument Management Handbook*, 29010831.

First-time use or after long-term storage

1. To remove ethanol, wash with 5 CV of distilled water at 0.1 ml/min (PC 1.6/5, 0.1 ml columns), 1 ml/min (5/50 GL, 1 ml and 4.6/100 PE, 1.7 ml columns), 2 ml/min (10/100 GL, 8 ml columns) or 4 ml/min (HR 16/10, 20 ml columns). This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 CV of start buffer at 0.1 ml/min (PC 1.6/5, 0.1 ml columns), 2 ml/min (5/50 GL, 1 ml and 4.6/100 PE, 1.7 ml columns), 4 ml/min (10/100 GL, 8 ml columns), or 8 ml/min (HR 16/10, 20 ml columns).
3. Wash with 5 CV of elution buffer, same flow as step 2.
4. Wash with 5 CV of start buffer, same flow as step 2.

Separation by gradient elution

Flow rates: 0.1 ml/min (PC 1.6/5, 0.1 ml columns), 2 ml/min (5/50 GL, 1 ml and 4.6/100 PE, 1.7 ml columns), 4 ml/min (10/100 GL, 8 ml columns) or 8 ml/min (HR 16/10, 20 ml columns). Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable, that is, when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10 to 20 CV and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 CV of 1 M NaCl (100%B) to elute any remaining ionically bound material.
6. Re-equilibrate with 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Although separations by step elution (see Chapter 1) can be performed using MonoBeads, gradient elution is recommended in order to achieve the highest possible resolution.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the column.

If ionic detergents have been used, wash the column with 5 CV of distilled water, followed by 2 CV of 2 M NaCl. Re-equilibrate with at least 10 CV of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove nonionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3. Refer to Chapter 2 for advice on optimizing the separation.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure, or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures to remove contaminants.

Reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter before trying alternative cleaning methods. Care should be taken when changing a filter as this can affect the column packing and interfere with performance.

Removing common contaminants

Flow rates: 0.05 ml/min (PC 1.6/5, 0.1 ml columns), 0.5 ml/min (5/50 GL, 1 ml columns), 0.2 ml/min (4.6/100 PE, 1.7 ml columns), 2 ml/min (10/100 GL, 8 ml columns), or 5 ml/min (HR 16/10, 20 ml columns).

1. Wash with at least 2 CV of 2 M NaCl.
2. Wash with at least 4 CV of 1 M NaOH.
3. Wash with at least 2 CV of 2 M NaCl.
4. Rinse with at least 2 CV of distilled water until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 CV of start buffer or storage buffer until pH and conductivity values have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins, refer to Appendix 1.

Chemical stability

For daily use, MonoBeads are stable in all common, aqueous buffers in the pH range 2 to 12, and in the presence of additives such as denaturing agents (8 M urea or 6 M guanidine hydrochloride), nonionic or ionic detergents, and up to 20% acetonitrile in aqueous buffers. Note that aqueous solutions of urea, ethylene glycol and similar compounds will increase the back pressure due to increased viscosity.

MonoBeads can be used with organic solutions such as dimethylsulfoxide, dimethylformamide, or formic acid, but the separation properties of the media will change.

Avoid anionic detergents with Mono Q. Avoid cationic detergents with Mono S. Avoid oxidizing agents.

Storage

For column storage, wash with 5 CV of distilled water followed by 5 CV of 20% ethanol. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid overpressuring the column. Store at room temperature or, for long periods, store at 4°C to 8°C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Do not freeze.

SOURCE: high-throughput, high-resolution purification, and easy scale-up

- Use SOURCE 15 for intermediate purification or polishing steps in laboratory- or large-scale applications that require high resolution and high throughput (flow velocities up to 1800 cm/h).
- Use SOURCE 30 as an alternative to SOURCE 15 for intermediate purification or polishing steps in large-scale applications where speed rather than resolution is a priority (flow velocities up to 2000 cm/h).
- Use SOURCE 30 as an alternative to SOURCE 15 for large sample volumes where speed rather than resolution is a priority. The larger particle size slightly reduces resolution, but separations can be performed at higher flow rates.
- Run SOURCE columns on ÄKTA chromatography systems, HPLC systems, or systems using peristaltic pumps. Appendix 4 provides guidance on selecting the right ÄKTA system.

SOURCE media are based on a hydrophilic matrix made from monodispersed, rigid, polystyrene/divinyl benzene and substituted with quaternary ammonium (Q) or methyl sulfonate (S) groups (Figure 3.13). This combination confers extreme chemical and physical stability to the media. The small particle sizes allow fast binding and dissociation to facilitate high resolution while the uniformity and stability of the particles ensures high flow rates at low back pressure. The strong ion exchange groups (Q and S) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application. The high flow rates that can be used with SOURCE media are more likely to be limited by the equipment available rather than the physical properties of the media.

Separation methods can be easily scaled up from columns such as RESOURCE Q or S, 1 ml prepacked with SOURCE 15, to large-scale columns such as FineLINE™.

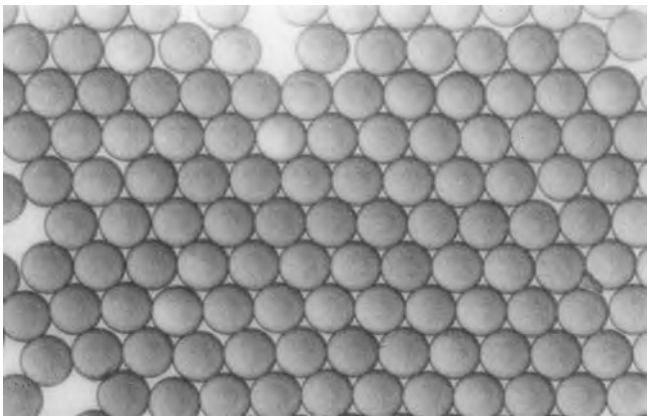


Fig 3.13. Uniform size distribution of SOURCE monodispersed particles.

Media characteristics

Composition: rigid, monodisperse, polystyrene/divinyl benzene particles with an optimized pore-size distribution. The base matrix is substituted with quaternary amino groups (Q) or methyl sulfonate groups (S), see Table 3.5.

Table 3.5. Characteristics of SOURCE 15 and 30 media

Product	Functional group	pH stability[1]	Mean particle size (µm, monosized)
SOURCE 15Q	$-CH_2-N^+-(CH_3)_3$	Long term: 2 to 12 Short term: 1 to 14	15
SOURCE 30Q	$-CH_2-N^+-(CH_3)_3$	Long term: 2 to 12 Short term: 1 to 14	30
SOURCE 15S	$-CH_2-SO_3^-$	Long term: 2 to 13 Short term: 1 to 14	15
SOURCE 30S	$-CH_2-SO_3^-$	Long term: 2 to 13 Short term: 1 to 14	30

[1] *Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance. Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. All ranges are estimates based on experience and knowledge gained at GE.*

Purification options

SOURCE Q and S media are available in media packs and in convenient prepacked Tricorn (PE) and RESOURCE columns (Fig 3.14). Purification options for the media and prepacked columns are described in Table 3.6.

Fig 3.14. SOURCE is available in media packs and is prepacked in Tricorn and RESOURCE columns.

Table 3.6. Purification options for SOURCE media and prepacked columns

Product, column volume	Binding capacity per column or per ml medium	Recommended working flow rate range[1]	Maximum flow[1]	Working pH range[2]	Maximum operating back pressure[3] (MPa/psi) *1 MPa = 10 bar*
Strong anion exchangers					
SOURCE 15Q	45 mg/ml (BSA, M_r 67 000)	150 to 900 cm/h	1800 cm/h	2 to 12	0.5/72
SOURCE 30Q	40 mg/ml (BSA, M_r 67 000)	300 to 1000 cm/h	2000 cm/h	2 to 12	0.5/72
RESOURCE Q, 1 ml	45 mg (BSA, M_r 67 000)	1.0 to 10 ml/min	10 ml/min	2 to 12	1.5/220
RESOURCE Q, 6 ml	270 mg (BSA, M_r 67 000)	1.0 to 60 ml/min	60 ml/min	2 to 12	0.6/87
SOURCE 15Q 4.6/100 PE, 1.7 ml	75 mg (BSA, M_r 67 000)	0.5 to 2.5 ml/min	5 ml/min	2 to 12	4/580
Strong cation exchangers					
SOURCE 15S	80 mg/ml (lysozyme, M_r 14 500)	150 to 900 cm/h	1800 cm/h	2 to 13	0.5/72
SOURCE 30S	80 mg/ml (lysozyme, M_r 14 500)	300 to 1000 cm/h	2000 cm/h	2 to 13	0.5/72
RESOURCE S, 1 ml	80 mg (lysozyme, M_r 14 500)	1.0 to 10 ml/min	10 ml/min	2 to 13	1.5/220
RESOURCE S, 6 ml	480 mg (lysozyme, M_r 14 500)	1.0 to 60 ml/min	60 ml/min	2 to 13	0.6/87
SOURCE 15S 4.6/100 PE, 1.7 ml	140 mg (lysozyme, M_r 14 500)	0.5 to 2.5 ml/min	5 ml/min	2 to 13	4/580

[1] *See Appendix 5 to convert flow velocity (cm/h) to volumetric flow rate (ml/min) and vice versa.*
[2] *Working pH range refers to the pH interval where the medium can be operated as intended without adverse long-term effects.*
[3] *Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

Use prepacked RESOURCE columns (1 ml or 6 ml) for fast media selection, method scouting, group separations, sample concentration or clean-up.

Use SOURCE 15Q PE 4.6/100 PE to improve resolution by increasing column length with further optimization and as the first step towards scaling up.

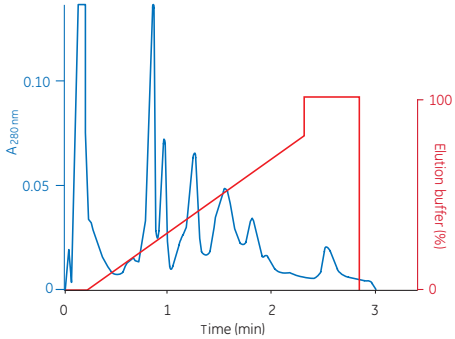
For column packing in XK columns, see Table 3.7. Select a production column such as FineLINE for larger volumes.

Table 3.7. Packing of SOURCE 15 and SOURCE 30 chromatography media in XK columns

	Volume (ml)	Bed height (cm)
SOURCE 15		
Tricorn 10/100	up to 8	up to 10
Tricorn 10/150	up to 12	up to 15
Tricorn 10/200	up to 16	up to 20
SOURCE 30		
XK 16/20	up to 30	up to 15
XK 26/20	up to 80	up to 15
XK 26/40	up to 196	> 15

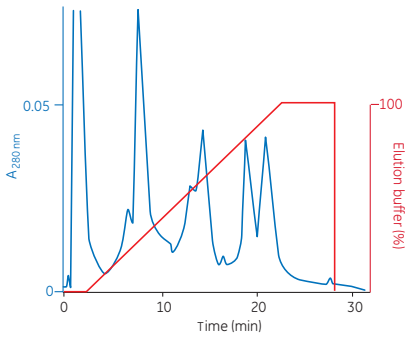
Purification examples

Fast, high-resolution separations



Column:	RESOURCE Q 1 ml
Sample:	Pancreatin 5 mg/ml
Sample volume:	200 µl
Start buffer:	20 mM bis-Tris-propane, pH 7.5
Elution buffer:	20 mM bis-Tris-propane, 500 mM NaCl, pH 7.5
Flow rate, flow velocity:	9.6 ml/min, 1800 cm/h
Gradient:	0% to 80% elution buffer in 20 CV

Fig 3.15. Separation of pancreatin on RESOURCE Q, 1 ml in 3 min.



Column:	RESOURCE S 1 ml
Sample:	Snake venom, 4 mg/ml
Sample volume:	100 µl
Start buffer:	20 mM sodium phosphate, pH 6.8
Elution buffer:	20 mM sodium phosphate, 400 mM NaCl, pH 6.8
Flow rate, flow velocity:	1 ml/min, 180 cm/h
Gradient:	0% to 100% elution buffer in 20 CV

Fig 3.16. Separation of snake venom on RESOURCE S, 1 ml at 1 ml/min (180 cm/h).

Scaling up: resolution maintained

Column:	(A) SOURCE 15S, 2.2 ml (B) SOURCE 15S, FineLINE 100, 390 ml
Sample:	Ribonuclease, cytochrome C and lysozyme
Sample load:	(A) 0.46 mg in 200 µl (B) 80.5 mg in 350 ml
Start buffer:	20 mM sodium phosphate, pH 6.8
Elution buffer:	20 mM sodium phosphate, 400 mM NaCl, pH 6.8
Flow rates (flow velocities):	(A) 2.2 ml/min (300 cm/h) (B) 385 ml/min (300 cm/h)
Gradient:	0% elution buffer (2 CV) 0% to 100% elution buffer (21 CV)

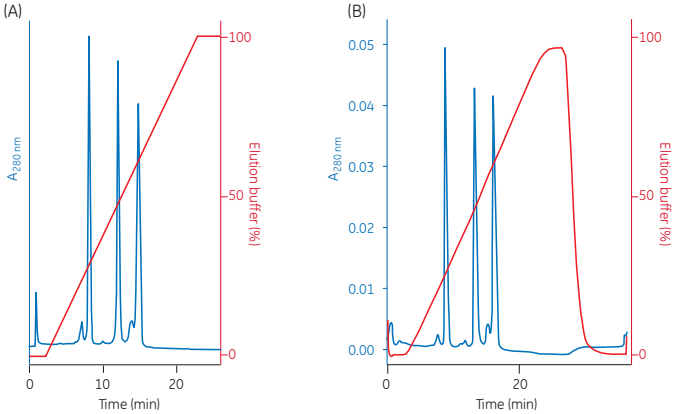
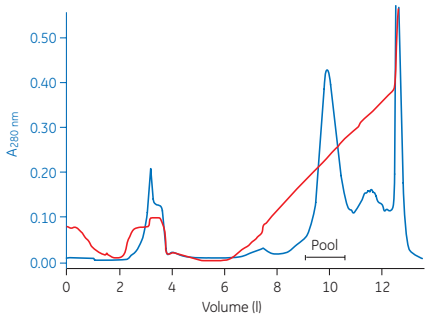


Fig 3.17. Separation of proteins scaled up from a 2.2 ml column to a 390 ml column.

Intermediate purification

Figure 3.18 shows an example of SOURCE 30Q used for an intermediate purification step in a large-scale process. Recombinant *P. aeruginosa* exotoxin A, produced as a periplasmic protein in *E. coli*, was initially purified with STREAMLINE DEAE expanded bed adsorption, followed by hydrophobic interaction chromatography (HIC) on Phenyl Sepharose 6 Fast Flow (high sub). The fraction of interest was then further purified on SOURCE 30Q before a final HIC polishing step on SOURCE 15PHE to remove the final contaminants.

Column:	SOURCE 30Q, FineLINE 100 (375 ml)
Sample:	Partially purified recombinant *P. aeruginosa* exotoxin A, diluted 1:3 with water
Sample:	1.8 g total protein (0.29 g exotoxin A) in 1.5 l
Start buffer:	20 mM sodium phosphate, pH 7.4
Elution buffer:	20 mM sodium phosphate, 1 M NaCl, pH 7.4
Flow rate (flow velocity):	785 ml/min (600 cm/h)
Gradient:	0% to 50% elution buffer in 20 CV



Native PAGE results, Coomassie staining
Lane 1. Pool from step 2 on Phenyl Sepharose Fast Flow (high sub)
Lane 2. Pool from step 3 on SOURCE 30Q
Lane 3. Pool from step 4 on SOURCE 15PHE

Fig 3.18. Intermediate purification of recombinant *Pseudomonas aeruginosa* exotoxin A on SOURCE 30Q.

Separations under extreme pH conditions

The high pH stability of SOURCE media makes them well-suited for applications requiring conditions of extreme pH such as purification of certain peptides and synthetic oligo-nucleotides, as shown in Figures 3.19 and 3.20.

Column:	RESOURCE S, 1 ml
Sample:	Partially purified bacitracin, 5 mg/ml
Sample volume:	100 µl
Start buffer:	5 mM potassium phosphate, pH 2.8, 30% acetonitrile
Elution buffer:	5 mM potassium phosphate, 400 mM KCl, pH 2.8, 30% acetonitrile
Flow rate (flow velocity):	1 ml/min (180 cm/h)
Gradient:	0% to 100% elution buffer in 10 CV

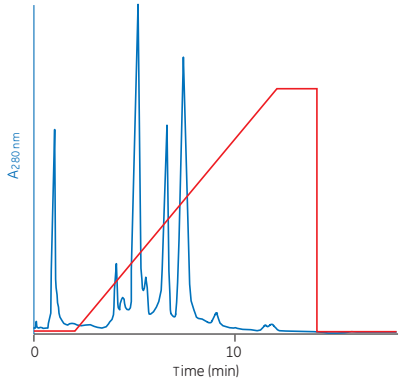


Fig 3.19. Intermediate purification of the peptide bacitracin from *Bacillus subtilis* on RESOURCE Q, 1 ml.

Method optimization

Sample: Crude oligonucleotide 20-mer (10 µg)
Start buffer: 10 mM NaOH, pH 12
Elution buffer: 10 mM NaOH, 2 M NaCl, pH 12
Flow rate: 1 ml/min

(A)
Column: RESOURCE Q, 1 ml
Gradient: 0% to 50% elution buffer in 30 CV

(B)
Column: RESOURCE Q, 1 ml
Gradient: 20% to 35% elution buffer in 50 CV

(C)
Column: SOURCE 15Q 4.6/100 PE, 1.7 ml
Gradient: 20% to 35% elution buffer in 50 CV

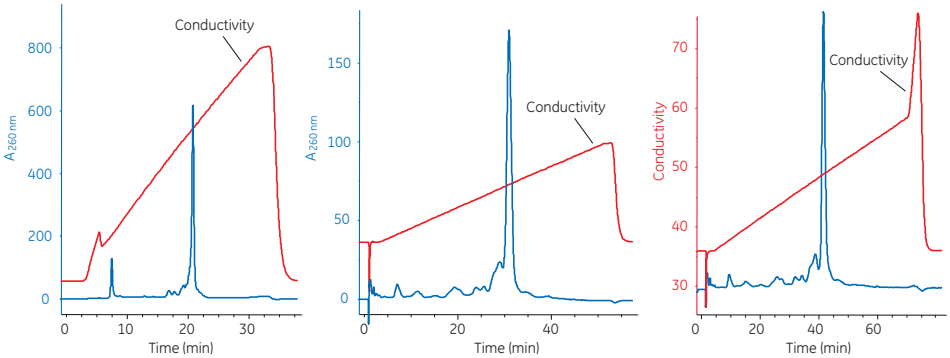


Fig 3.20. Manipulation of gradient slope and shape to maximize resolution. Initial purification of a 20 mer oligonucleotide was optimized on RESOURCE Q, 1 ml and transferred to SOURCE Q PE 4.6/100 to further increase resolution by increasing bed height to 10 cm.

Batch-to-batch reproducibility

Batch-to-batch reproducibility is particularly important for media used for scaling-up and large scale industrial applications which are under strict regulatory control. Figures 3.21 and 3.22 demonstrate the high batch-to-batch reproducibility of SOURCE 15 and SOURCE 30 media.

Columns: SOURCE 30S, 2.2 ml, four separate batches
Sample: Chymotrypsinogen, cytochrome C, and lysozyme
Sample load: 0.32 mg/ml bed volume
Start buffer: 20 mM sodium phosphate, pH 6.8
Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, pH 6.8
Flow rate (flow velocity): 2.2 ml/min (300 cm/h)
Gradient: 0% to 100% elution buffer in 20 CV

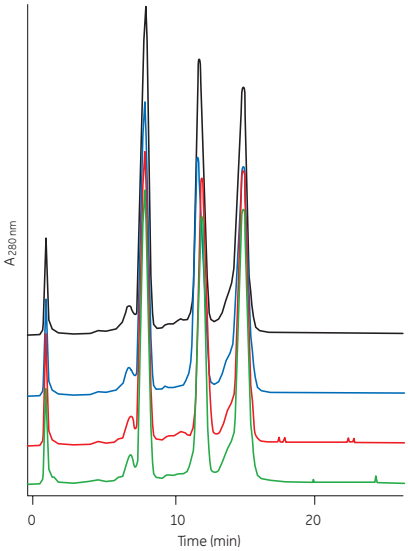


Fig 3.21. Selectivity tests on four production batches of SOURCE 30S.

Columns:	SOURCE 15Q, 2.2 ml, four separate batches
Sample:	Ovalbumin (3 mg/ml), β-lactoglobulin (3 mg/ml)
Sample volume:	200 µl
Start buffer:	20 mM bis-Tris-propane, pH 7.0
Elution buffer:	20 mM bis-Tris-propane, 350 mM NaCl, pH 7.0
Flow rate (flow velocity):	2.2 ml/min (300 cm/h)
Gradient:	0% elution buffer (2 CV) 0% to 100% elution buffer (21 CV)

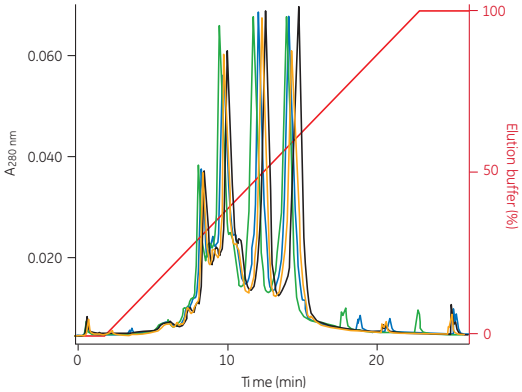


Fig 3.22. Selectivity tests on four production batches of SOURCE 15Q.

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions, and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions using filters of 0.45 µm or 0.22 µm for 30 µm particles and 0.22 µm filters for 15 µm particles. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

The pH of the start buffer should be at least 0.5 to 1.0 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5 to 1.0 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and nonvolatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

anion exchange (Q)

start buffer: 20 mM Tris-HCl, pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

cation exchange (S)

start buffer: 20 mM 2-(N-Morpholino)ethanesulfonic acid (MES), pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTA systems with automatic buffer preparation functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8.0 or cation exchange chromatography at pH 6.0, see *ÄKTA Laboratory-scale Chromatography Systems: Instrument Management Handbook*, 29010831.

First-time use or after long-term storage

1. To remove ethanol, wash with 5 CV of distilled water at 2 ml/min (SOURCE 15 4.6/100 PE), 4 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml), or 200 cm/h for SOURCE packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 CV of start buffer, at 2 ml/min (SOURCE 15 4.6/100 PE), 4 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml), or 200 cm/h for SOURCE packed in larger columns.
3. Wash with 5 CV of elution buffer, same flow as step 2.
4. Wash with 5 CV of start buffer, same flow as step 2.

Separation by gradient elution

Flow rates: 2 ml/min (SOURCE 15 4.6/100 PE), 4 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml), or 200 cm/h for SOURCE packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable, that is, when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10 to 20 CV and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 CV of 1 M NaCl (100%B) to elute any remaining ionically bound material.
6. Re-equilibrate with 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Flow rates: 2 ml/min (SOURCE 15 4.6/100 PE), 4 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml), or 200 cm/h for SOURCE packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH, and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable, that is, when all unbound material has washed through the column.
4. Elute the target protein with 5 CV of start buffer containing NaCl at chosen ionic strength.
5. If necessary: repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 CV of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically bound material.
7. Re-equilibrate with 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

If ionic detergents have been used, wash the column with 5 CV of distilled water, followed by 2 CV of 2 M NaCl. Re-equilibrate with at least 10 CV of start buffer until the UV baseline, eluent pH, and/or conductivity are stable. Organic solvents such as ethanol can be used to remove nonionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

Reverse the direction of flow during column cleaning so that contaminatants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step varies according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this can affect the column packing and interfere with performance.

Removing common contaminants

Flow rates: 0.2 ml/min (SOURCE 15 4.6/100 PE), 1 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml), or 40 cm/h with a contact time of 1 to 2 h for SOURCE packed in larger columns

1. Wash with at least 2 CV of 2 M NaCl.
2. Wash with at least 4 CV of 1 M NaOH.
3. Wash with at least 2 CV of 2 M NaCl.
4. Rinse with at least 2 CV of distilled water until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 CV of start buffer or storage buffer until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins, refer to Appendix 1.

Chemical stability

For daily use, SOURCE media are stable in all common, aqueous buffers pH 2 to 12, denaturing agents (8 M urea, 6 M guanidine hydrochloride), 75% acetic acid, 1 M NaOH, 1 M HCl, 70% ethanol, 30% acetonitrile, and with additives such as nonionic detergents.

Avoid cationic detergents with SOURCE S. Avoid anionic detergents with SOURCE Q. Avoid oxidizing agents.

Storage

For column storage, wash with 5 CV of distilled water followed by 5 CV of 20% ethanol. Include 200 mM sodium acetate in the 20% ethanol solution for SOURCE S. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid overpressuring the column. Store at room temperature or, for long periods, store at 4°C to 8°C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at 4°C to 30°C in 20% ethanol. Do not freeze.

Sepharose High Performance: purification with high resolution

Use Sepharose High Performance for intermediate purification steps that require high capacity and high resolution (flow velocities up to 150 cm/h).

Run Sepharose High Performance columns on ÄKTA chromatography systems. Appendix 4 provides guidance on selecting the right ÄKTA system.

Sepharose High Performance media are based on a matrix of 34 µm particles made from 6% agarose and highly cross-linked for chemical and physical stability. The small particle size ensures fast binding and dissociation even at high sample loads and flow rates which, in combination with high selectivity, give high-resolution separations. Particle size and bed volumes remain stable, despite changes in ionic strength or pH, to ensure fast separations at high flow rates. The strong ion exchange groups (Q and SP) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Media characteristics

Composition: sulfopropyl (SP) or quaternary amino (Q) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds, see Table 3.8.

Table 3.8. Characteristics of Sepharose High Performance media

Product	Functional group	pH stability[1]	Mean particle size (µm)
Q Sepharose High Performance	$-CH_2-N^+-(CH_3)_3$	Long term: 2 to 12 Short term: 1 to 14	34
SP Sepharose High Performance	$-CH_2-SO_3^-$	Long term: 4 to 13 Short term: 3 to 14	34

[1] *Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on chromatography performance. Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. All ranges are estimates based on experience and knowledge gained at GE.*

Purification options

Sepharose Q and SP High Performance are available in chromatography media packs, in convenient prepacked HiTrap columns for small-scale work, HiPrep columns for purification scale-up, and HiScreen columns (Fig 3.23). The purification options for these prepacked formats are found in Table 3.9.

Fig 3.23. Q and SP Sepharose High Performance media are available prepacked in HiTrap, HiPrep, and HiScreen columns or in media packs.

Table 3.9. Purification options for Sepharose High Performance media and prepacked columns

Product	Binding capacity per column or per ml medium	Recommended working flow[1]	Maximum flow[1]	Working pH range[2]	Maximum operating back pressure[3] (MPa/psi) *1 MPa = 10 bar*
Strong anion exchangers					
Q Sepharose High Performance	70 mg/ml (HSA, M_r 68 000)	30 to 150 cm/h	150 cm/h	2 to 12	0.5/72
HiTrap Q HP, 1 ml	50 mg (HSA, M_r 68 000)	up to 1 ml/min	4 ml/min	2 to 12	0.3/43
HiTrap Q HP, 5 ml	250 mg (HSA, M_r 68 000)	up to 5 ml/min	20 ml/min	2 to 12	0.3/43
HiPrep Q HP, 20 ml	1000 mg (BSA, M_r 68 000)	up to 5 ml/min	20 ml/min	2 to 12	
HiScreen Q HP, 4.7 ml	70 mg/ml (BSA, M_r 68 000)	0.6 ml/min	1.2 ml/min	2 to 12	0.3/43
Strong cation exchangers					
SP Sepharose High Performance	55 mg/ml (ribonuclease, M_r 13 700)	30 to 150 cm/h	150 cm/h	4 to 13	0.5/72
HiTrap SP HP, 1 ml	55 mg (ribonuclease, M_r 13 700)	up to 1 ml/min	4 ml/min	4 to 13	0.3/43
HiTrap SP HP, 5 ml	275 mg (ribonuclease, M_r 13 700)	up to 5 ml/min	20 ml/min	4 to 13	0.3/43
HiPrep SP HP 16/10, 20 ml	1100 mg (ribonuclease, M_r 13 700)	up to 5 ml/min	20 ml/min	4 to 13	
HiScreen S HP, 4.7 ml	55 mg/ml (ribonuclease, M_r 13 500)	0.6 ml/min	1.2 ml/min	4 to 12	0.3/43

[1] *See Appendix 5 to convert flow velocity (cm/h) to volumetric flow rate (ml/min) and vice versa.*
[2] *Working pH range refers to the pH interval where the medium can be operated as intended without adverse long-term effects.*
[3] *Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

- Use prepacked HiTrap columns (1 ml or 5 ml) for media selection, method scouting, group separations, small-scale purification, sample concentration, or clean-up. Connect up to three HiTrap columns in series to scale-up.
- Use prepacked HiPrep columns (20 ml) for method development, group separations, larger scale purification, or sample concentration.
- Use prepacked HiScreen columns (4.7 ml, bed height 10 cm) for method development and screening before scaling up with bed height maintained.

For column packing in Tricorn and XK columns, see Table 3.10.

Table 3.10. Packing volumes and bed heights for Sepharose Q and SP High Performance media packed in Tricorn and XK columns

Column	Volume (ml)	Bed height (cm)
Tricorn 10/100	up to 8	up to 10
Tricorn 10/150	up to 12	up to 15
Tricorn 10/200	up to 16	up to 20
XK 16/20	up to 30	up to 15
XK 26/20	up to 80	up to 15
XK 26/40	up to 196 ml	> 15 cm

Purification examples

Figure 3.24 shows scaling up from HiTrap SP HP to HiPrep SP HP 16/10. A number of factors were kept constant, such as sample load/ml medium, flow rates and number of CV in the gradient. The separation was maintained through a 20-fold scale-up. The scale-up resulted in similar separation of the sample, which comprised four standard proteins.

HiTrap SP HP, 1 ml

Sample:	Concanavalin A, ribonuclease A, α-chymotrypsinogen A, lysozyme, 4 mg protein/ml (3:3:1:1) in start buffer
Sample load:	1 mg protein/ml medium
Sample volume:	0.25 ml, 25% of CV
Flow rate (flow velocity):	0.5 ml/min (75 cm/h)
Start buffer:	50 mM MES, pH 6.0
Elution buffer:	50 mM MES, 1 M NaCl, pH 6.0
Gradient:	0% to 43% elution buffer over 10 ml (10 CV)

HiPrep SP HP 16/10, 20 ml

Sample:	Concanavalin A, ribonuclease A, α-chymotrypsinogen A, lysozyme, 4 mg protein/ml (3:3:1:1) in start buffer
Sample load:	1 mg protein/ml medium
Sample volume:	5.0 ml, 25% of CV
Flow rate (flow velocity):	2.5 ml/min (75 cm/h)
Start buffer:	50 mM MES, pH 6.0
Elution buffer:	50 mM MES, 1 M NaCl, pH 6.0
Gradient:	0% to 43% elution buffer over 200 ml (10 CV)

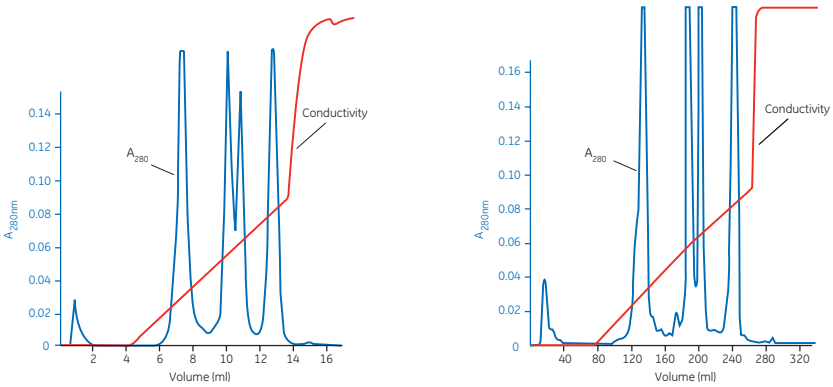
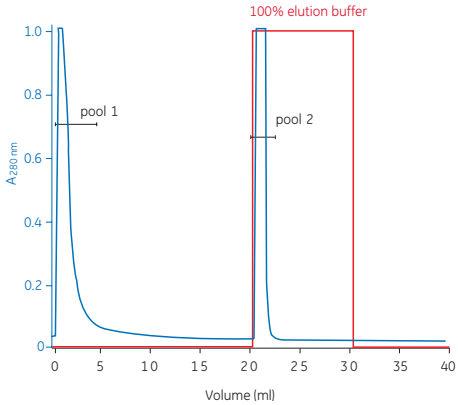


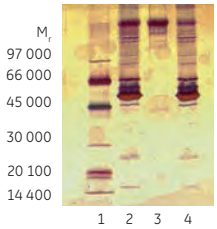
Fig. 3.24. Scaling up the separation of four standard proteins from HiTrap SP to HiPrep SP HP 16/10.

Group separations

Figure 3.25 shows a group separation of human serum proteins on HiTrap Q HP using a one-step elution that had been optimized to ensure that IgG flowed through the column leaving other serum components to be eluted separately.

Column:	HiTrap Q HP 1 ml
Sample:	Human serum, filtered (0.45 µm filter) and buffer exchanged to start buffer on a PD-10 Desalting column
Sample volume:	1.0 ml
Flow rate (flow velocity):	0.5 ml/min (75 cm/h)
Start buffer:	75 mM Tris-HCl, pH 8.0
Elution buffer:	75 mM Tris-HCl, 1.0 M NaCl, pH 8.0





Lane 1. Low Molecular Weight (LMW) Calibration Kit, GE Healthcare
Lane 2. Start material, buffer exchanged human serum, diluted 1:75
Lane 3. Flowthrough, pool 1, diluted 1:10
Lane 4. Desorbed material, pool 2, diluted 1:25

Fig 3.25. Separation of IgG from human serum proteins on HiTrap Q HP 1 ml, using one-step elution. Analysis by SDS-PAGE (silver staining).

Sample concentration

Concentrating a sample prior to SEC minimizes sample volume and facilitates a rapid, high-resolution size separation. HiTrap columns offer a convenient, ready-to-use solution for sample concentration. Table 3.11 gives examples of the high concentration factors achieved when concentrating proteins from very dilute starting material using HiTrap columns prepacked with Sepharose HP medium. Similar results can be achieved with HiTrap columns prepacked with Sepharose Fast Flow or Sepharose XL media.

Table 3.11. Sample concentration using 1 ml HiTrap ion exchange columns

Column	Sample	Sample concentration (µg/ml)	Sample volume (ml)	Eluted concen-tration (µg/ml)	Volume eluted (ml)	Concentration factor (volume)	Yield (%)
HiTrap Q HP, 1 ml	Human IgG	23	450	3180	3.0	150	92
		10	100	4700	2.0	50	93
		1010	10	3370	3.0	3	100
HiTrap SP HP, 5 ml	Lysozyme	333	150	3170	16.0	9	100
		33	1500	3720	13.2	114	98

Performing a separation

Guidelines for selection of media, buffer, pH, and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high-quality water and chemicals. Filter solutions through 0.45 µm or 0.22 µm filters. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

The pH of the start buffer should be at least 0.5 to 1.0 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5 to 1.0 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and nonvolatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

anion exchange (Q)

start buffer: 20 mM Tris-HCl, pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

cation exchange (S)

start buffer: 20 mM 2-(N-Morpholino)ethanesulfonic acid (MES), pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTA systems with automatic buffer preparation functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8.0 or cation exchange chromatography at pH 6.0, see *ÄKTA Laboratory-scale Chromatography Systems: Instrument Management Handbook*, 29010831.

First-time use or after long-term storage

1. To remove ethanol, wash with 1 CV of distilled water at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 0.6 ml/min (HiScreen 4.7 ml), 0.8 ml/min (HiPrep 20 ml), or at 25 cm/h for Sepharose High Performance packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 CV of start buffer at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml) 0.6 ml/min (HiScreen 4.7 ml), 3 ml/min (HiPrep 20 ml) or at 50 cm/h for Sepharose High Performance packed in larger columns.
3. Wash with 5 CV of elution buffer, same flow as step 2.
4. Wash with 5 CV of start buffer, same flow as step 2.
5. Run a blank elution before applying sample.

Separation by gradient elution

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 0.6 ml/min (HiScreen 4.7 ml), 3 ml/min (HiPrep 20 ml), or at 50 to 100 cm/h for Sepharose High Performance packed in larger columns. Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable, that is, when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10 to 20 CV and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 CV of 1 M NaCl (100%B) to elute any remaining ionically bound material.
6. Re-equilibrate with 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiPrep 20 ml), 0.6 ml/min (HiScreen 4.7 ml), or at 50 to 100 cm/h for Sepharose High Performance packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivityare stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH and conductivity are stable, that is, when all unbound material has washed through the column.
4. Elute the target protein with 5 CV of start buffer containing NaCl at chosen ionic strength.
5. If necessary: repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 CV of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically bound material.
7. Re-equilibrate with 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

If ionic detergents have been used, wash the column with 5 CV of distilled water, followed by 2 CV of 2 M NaCl. Re-equilibrate with at least 10 CV of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove nonionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3. Note that this does not apply to HiTrap columns.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indicators that the medium needs to be cleaned using more stringent procedures to remove contaminants.

Reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step varies according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this can affect column packing and interfere with performance.

Removing common contaminants

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 0.6 ml/min (HiScreen 4.7 ml), 3 ml/min (HiPrep 20 ml), or at 40 cm/h with a contact time of 1 to 2 h for Sepharose High Performance packed in larger columns.

1. Wash with at least 2 CV of 2 M NaCl at 0.6 ml/min (HiScreen 4.7 ml) 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 3 ml/min (HiPrep 20 ml), or at 40 cm/h with a contact time of 1 to 2 h for Sepharose High Performance packed in larger columns.
2. Wash with at least 4 CV of 1 M NaOH.
3. Wash with at least 2 CV of 2 M NaCl.
4. Rinse with at least 2 CV of distilled water until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 CV of start buffer or storage buffer until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins, refer to Appendix 1.

Chemical stability

For daily use, Sepharose High Performance media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), 70% ethanol, 1 M acetic acid, 30% acetonitrile, and with additives such as nonionic detergents.

Sepharose High Performance can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane, and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.

Avoid cationic detergents with SP Sepharose High Performance. Avoid anionic detergents with Q Sepharose High Performance. Avoid oxidizing agents.

Storage

For column storage, wash with 2 CV of distilled water followed by 2 CV of 20% ethanol. Include 0.2 M sodium acetate in the 20% ethanol solution for columns packed with SP Sepharose High Performance. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid overpressuring the column. Store at room temperature or, for long periods, store at 4°C to 8°C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at 4°C to 30°C in 20% ethanol. Do not freeze.

To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

Sepharose Fast Flow: purification with good resolution and easy scale-up

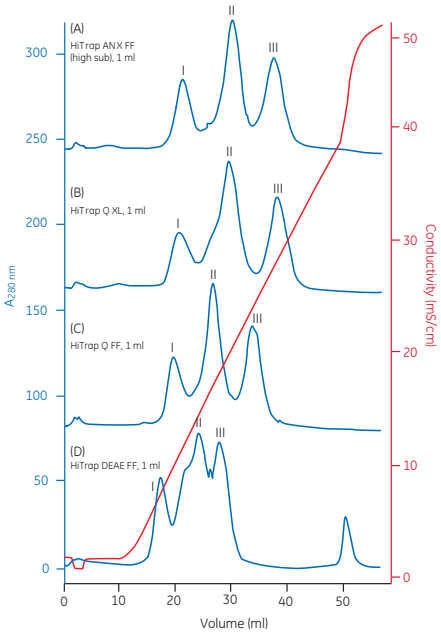
- Use Sepharose Fast Flow for capture or intermediate purification steps that require good resolution (flow velocity up to 300 cm/h).
- Use a weak ion exchanger such as DEAE, CM, or ANX Sepharose Fast Flow, if a strong ion exchanger (substituted with Q or SP) does not give the required selectivity.
- Run Sepharose Fast Flow columns on ÄKTA chromatography systems, HPLC systems, or systems using peristaltic pumps. Appendix 4 provides guidance on selecting the right ÄKTA system.

Sepharose Fast Flow media are based on a matrix of 90 µm particles made from 6% agarose and highly cross-linked for chemical and physical stability. ANX Sepharose 4 Fast Flow (high sub) is based on 4% agarose to form a medium that maintains a high binding capacity when separating large molecules such as thyroglobulin (M_r = 650 000), particularly suitable for large-scale production when total binding capacity becomes economically significant.

Sepharose Fast Flow matrices are substituted with a range of ion exchange groups (Q, DEAE, ANX, SP, and CM) giving the opportunity to test and use different selectivities (see Chapter 1 for an explanation of strong and weak ion exchangers). Ion exchangers containing strong ion exchange groups (Q and SP) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Ion exchangers containing weak ion exchange groups (DEAE, CM, and ANX) offer alternative selectivities, but over a narrower pH working range. Figure 3.26 illustrates how the selectivity of Sepharose Fast Flow media changes according to the anion exchange group.

Particle size and bed volumes remain stable, despite changes in ionic strength or pH, to ensure fast separations at high flow rates with good resolution. Methods can be easily scaled up from columns such as HiTrap Q FF (1 ml, prepacked with Q Sepharose Fast Flow) through to large-scale columns such as FineLINE. The performance of Sepharose Fast Flow is well documented and there are many examples of the smooth transfer from the laboratory to pilot scale and on to production.



Columns: (A) HiTrap ANX FF (high sub), 1 ml
(B) HiTrap Q XL, 1 ml
(C) HiTrap Q FF, 1 ml
(D) HiTrap DEAE FF, 1 ml

Sample: 0.4 mg conalbumin (pI = 6.3), 0.8 mg α-lactoglobulin (pI = 5.8), 1.2 mg soya bean trypsin inhibitor (pI = 4.5) dissolved in 2 ml start buffer

Sample volume: 2 ml

Start buffer: 20 mM Tris-HCl pH 7.4

Elution buffer: 20 mM Tris-HCl, 500 mM NaCl pH 7.4

Flow rate: 1 ml/min (150 cm/h)

Gradient: 0% elution buffer (25 CV), 0% to 80% elution buffer (40 CV)

Wash: 5 ml start buffer

Elution: 40 ml, linear gradient, 0% to 80% elution buffer

Fig 3.26. Separation of conalbumin (I), α-lactalbumin (II) and soya bean trypsin inhibitor (III) on a range of anion exchange HiTrap columns demonstrates the difference in selectivity according to the anion exchange group.

Media characteristics

Composition:

- Sulfopropyl (SP), carboxymethyl (CM), quaternary amino (Q) or diethylaminoethyl (DEAE) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds
- Diethylaminopropyl (ANX) group coupled to highly cross-linked 4% agarose via chemically stable ether bonds

The characteristics of Sepharose Fast Flow media are shown in Table 3.12.

Table 3.12. Characteristics of Sepharose Fast Flow media

Product	Functional group	pH stability[1]	Mean particle size (µm)
Q Sepharose Fast Flow	$-CH_2-N^+-(CH_3)_3$	Long term: 2 to 12 Short term: 2 to 14	90
SP Sepharose Fast Flow	$-CH_2-CH_2-CH_2-SO_3^-$	Long term: 4 to 13 Short term: 3 to 14	90
DEAE Sepharose Fast Flow	$-CH_2-CH_2-N^+-(CH_2-CH_3)_2$	Long term: 2 to 12 Short term: 2 to 14	90
ANX Sepharose 4 Fast Flow	$-CH_2-CHOH-CH_2-N^+-(CH_2-CH_3)_2$	Long term: 3 to 13 Short term: 2 to 14	90
CM Sepharose Fast Flow	$-CH_2-COO^-$	Long term: 4 to 13 Short term: 2 to 14	90

[1] *Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance. Short-term, pH stability refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. All ranges are estimates based on experience and knowledge gained at GE.*

Purification options

Sepharose Fast Flow media, with a range of selectivities, are available prepacked in HiTrap, HiScreen, and HiPrep columns and in media packs (Fig 3.27). Purification options for the media and prepacked columns are shown in Table 3.13.

Fig 3.27. The range of Sepharose Fast Flow is available in media packs and prepacked HiTrap, HiPrep, and HiScreen columns. The media are also available in high-throughput process development (HTPD) format, see Chapter 5, *Large-scale purification*.

Table 3.13. Purification options for Sepharose Fast Flow media and prepacked columns

Product	Binding capacity per column or per ml medium	Recommended working flow[1]	Maximum flow[1]	Working pH range[2]	Maximum operating back pressure[3] (MPa/psi) *1 MPa = 10 bar*
Strong anion exchangers					
Q Sepharose Fast Flow	3 mg/ml (thyroglobulin, M_r 669 000) 120 mg/ml (HSA, M_r 68 000) 110 mg/ml, (α–lactalbumin, M_r 14 300)	50 to 400 cm/h	750 cm/h	2 to 12	0.3/43
HiTrap Q FF, 1 ml	3 mg (thyroglobulin, M_r 669 000) 120 mg (HSA, M_r 68 000) 110 mg (α–lactalbumin, M_r 14 300)	up to 1 ml/min	4 ml/min	2 to 12	0.3/43
HiTrap Q FF, 5 ml	15 mg (thyroglobulin, M_r 669 000) 600 mg (HSA, M_r 68 000) 550 mg (α–lactalbumin, M_r 14 300)	up to 5 ml/min	20 ml/min	2 to 12	0.3/43
HiScreen Q FF, 4.7 ml	120 mg/ml (HSA, M_r 68 000)	2.3 ml/min	3.5 ml/min	2 to 12	0.15/22
HiPrep Q FF 16/10, 20 ml	60 mg (thyroglobulin, M_r 669 000) 2400 mg (HSA, M_r 68 000) 2200 mg (α–lactalbumin, M_r 14 300)	2 to 10 ml/min	10 ml/min	2 to 12	0.15/22
Weak anion exchangers					
DEAE Sepharose Fast Flow	100 mg/ml (α–lactalbumin, M_r 14 300) 110 mg/ml (HSA, M_r 68 000)	50 to 400 cm/h	750 cm/h	2 to 9	0.3/43
ANX Sepharose 4 Fast Flow (high sub)	43 mg/ml (BSA, M_r 67 000) 5 mg/ml (thyroglobulin, M_r 669 000)	50 to 300 cm/h	400 cm/h	3 to 10	0.1/14
HiTrap DEAE FF, 1 ml	100 mg (α–lactalbumin, M_r14 300) 110 mg (HSA, M_r 68 000)	up to 1 ml/min	4 ml/min	2 to 9	0.3/43
HiTrap DEAE FF, 5 ml	500 mg (α–lactalbumin, M_r 14 300) 550 mg (HSA, M_r 68 000)	up to 5 ml/min	20 ml/min	2 to 9	0.3/43
HiScreen DEAE FF	110 mg (HSA, M_r 68 000)	2.3 ml/min	3.5 ml/min	2 to 9	0.15/22
HiPrep DEAE FF 16/10, 20 ml	2000 mg (α–lactalbumin, M_r 14 300) 2200 mg (HSA, M_r 68 000)	2 to 10 ml/min	10 ml/min	2 to 9	0.15/22
HiTrap ANX FF (high sub), 1 ml	43 mg (BSA, M_r 67 000) 5 mg (thyroglobulin, M_r 669 000)	up to 1 ml/min	4 ml/min	3 to 10	0.3/43
HiTrap ANX FF (high sub), 5 ml	215 mg (BSA, M_r 67 000) 25 mg (thyroglobulin, M_r 669 000)	up to 5 ml/min	20 ml/min	3 to 10	0.3/43
Strong cation exchangers					
SP Sepharose Fast Flow	50 mg/ml (bovine COHb, M_r 69 000) 50 mg/ml (human IgG, M_r 160 000) 70 mg/ml (ribonuclease A, M_r 13 700)	50 to 400 cm/h	750 cm/h	4 to 13	0.3/43
HiTrap SP FF, 1 ml	50 mg (bovine COHb, M_r 69 000) 50 mg (human IgG, M_r 160 000) 70 mg (ribonuclease A, M_r 13 700)	up to 1 ml/min	4 ml/min	4 to 13	0.3/43
HiTrap SP FF, 5 ml	250 mg (bovine COHb, M_r 69 000) 250 mg (human IgG, M_r 160 000) 350 mg (ribonuclease A, M_r 13 700)	up to 5 ml/min	20 ml/min	4 to 13	0.3/43
HiScreen SP FF, 4.7 ml	110 mg/ml (HSA, M_r 68 000)	2.3 ml/min	3.5 ml/min	4 to 13	0.15/22
HiPrep SP FF 16/10, 20 ml	1000 mg (bovine COHb, M_r 69 000) 1000 mg (human IgG, M_r 160 000) 1400 mg (ribonuclease A, M_r 13 700)	2 to 10 ml/min	10 ml/min	4 to 13	0.15/22

Continues on following page

Table 3.13 cont.

Product	Binding capacity per column or per ml medium	Recommended working flow[1]	Maximum flow[1]	Working pH range[2]	Maximum operating back pressure[3] (MPa/psi) *1 MPa = 10 bar*
Weak cation exchangers					
CM Sepharose Fast Flow	50 mg/ml medium (ribonuclease A, M_r 13 700)	50 to 400 cm/h	750 cm/h	6 to 10	0.3/43
HiTrap CM FF, 1 ml	50 mg (ribonuclease A, M_r 13 700)	up to 1 ml/min	4 ml/min	6 to 10	0.3/43
HiTrap CM FF, 5 ml	250 mg (ribonuclease A, M_r 13 700)	up to 5 ml/min	20 ml/min	6 to 10	0.3/43
HiPrep CM FF 16/10, 20 ml	1000 mg (ribonuclease A, M_r 13 700)	2 to 10 ml/min	10 ml/min	6 to 10	0.15/22

[1] *See Appendix 5 to convert flow velocity (cm/h) to volumetric flow rate (ml/min) and vice versa.*
[2] *Working pH range refers to the pH interval where the medium can be operated as intended without adverse long-term effects.*
[3] *Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

- Use prepacked HiTrap columns (1 ml or 5 ml) for media selection, method scouting, group separations, small-scale purification, sample concentration, or clean-up. Connect up to three HiTrap columns in series to scale-up.
- Use prepacked HiPrep columns (20 ml) for method development, group separations, larger scale purification, sample concentration or clean-up. Connect several HiPrep columns in series to increase binding capacity.
- Use prepacked HiScreen columns (4.7 ml, bed height 10 cm) for method development and screening before scaling up with bed height maintained.

For column packing in Tricorn and XK columns, see Table 3.14. Select a production column such as BPG or Chromaflow for larger volumes.

Table 3.14. Packing volumes and bed heights for Sepharose Fast Flow media packed in Tricorn and XK columns

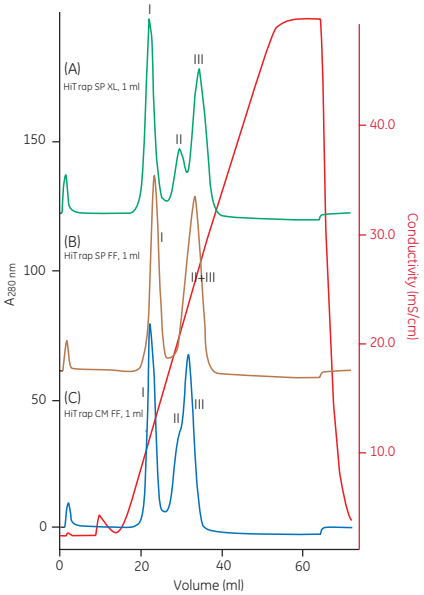
Column	Volume (ml)	Bed height (cm)
Tricorn 10/100	up to 8	up to 10
Tricorn 10/150	up to 12	up to 15
Tricorn 10/200	up to 16	up to 20
XK 16/20	up to 30	up to 15
XK 26/20	up to 80	up to 15
XK 26/40	up to 196	> 15
XK 50/20	up to 274	up to 14
XK 50/30	up to 559	up to 28.5

Purification examples

Media scouting

Using 1 ml HiTrap columns the most suitable matrix and charged group for a separation can be quickly and easily selected before optimization and scale-up. In Figure 3.28, a comparison of elution profiles for the same sample separated under identical conditions on three different Sepharose media illustrates the differences in selectivity and resolution that can result from changing the charge group and the particle size. The most suitable medium can be selected and conditions optimized according to the requirements for the separation, for example to isolate a single, well-resolved peak or to maximize resolution between several peaks of interest.

Begin by scouting on the strong ion exchangers (Q, S, or SP) in order to find the greatest differences in charge between the molecules of interest.

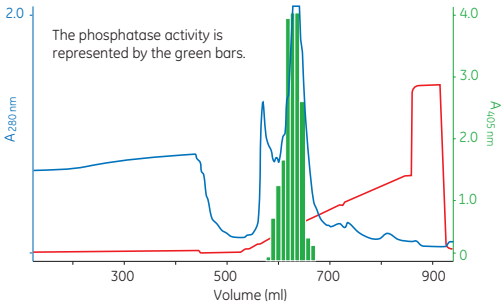


Columns:	(A) HiTrap SP XL, 1 ml (B) HiTrap SP FF, 1 ml (C) HiTrap CM FF, 1 ml
Sample:	3 mg ribonuclease A (pI = 9.3), 0.8 mg cytochrome C (pI = 10.3), 0.8 mg lysozyme (pI > 11.0)
Sample volume:	2 ml in start buffer
Start buffer:	20 mM sodium phosphate, pH 6.8
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl pH 6.8
Flow rate (flow velocity):	1 ml/min (150 cm/h)
Gradient:	0% elution buffer (25 CV), 0% to 100% elution buffer (40 CV)

Fig 3.28. Media scouting: separation of ribonuclease A (I), cytochrome C (II), and lysozyme (III) on HiTrap CM FF 1 ml, HiTrap SP FF 1 ml, and HiTrap SP XL 1 ml.

Capture

An example of capture using Sepharose DEAE Fast Flow medium prepacked in HiPrep DEAE FF 16/10 is shown in Figure 3.29.



Column:	HiPrep DEAE FF 16/10, 20 ml
Sample:	200 ml clarified *E. coli* supernatant, diluted 1:2 with water, pH 6.6, conductivity 2.6 mS/cm
Start buffer:	25 mM Tris-HCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, pH 7.4
Elution buffer:	1 M NaCl, 25 mM Tris-HCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, pH 7.4
Flow rate (flow velocity):	5 ml/min (150 cm/h)
Gradient:	0% elution buffer (6 CV) 0% to 50% elution buffer (20 CV) 50% elution buffer (1 CV) 100% elution buffer (2 CV)

Fig 3.29. A HiPrep DEAE FF 16/10 column is used as the capture step to concentrate rPhosphatase and remove most of the contaminants.

Scaling-up

Figure 3.30 shows the ease with which separations can be scaled up on columns prepacked with Sepharose Fast Flow. Beginning with a 1 ml HiTrap column the reproducibility of the separation has been maintained through a 20-fold scale-up.

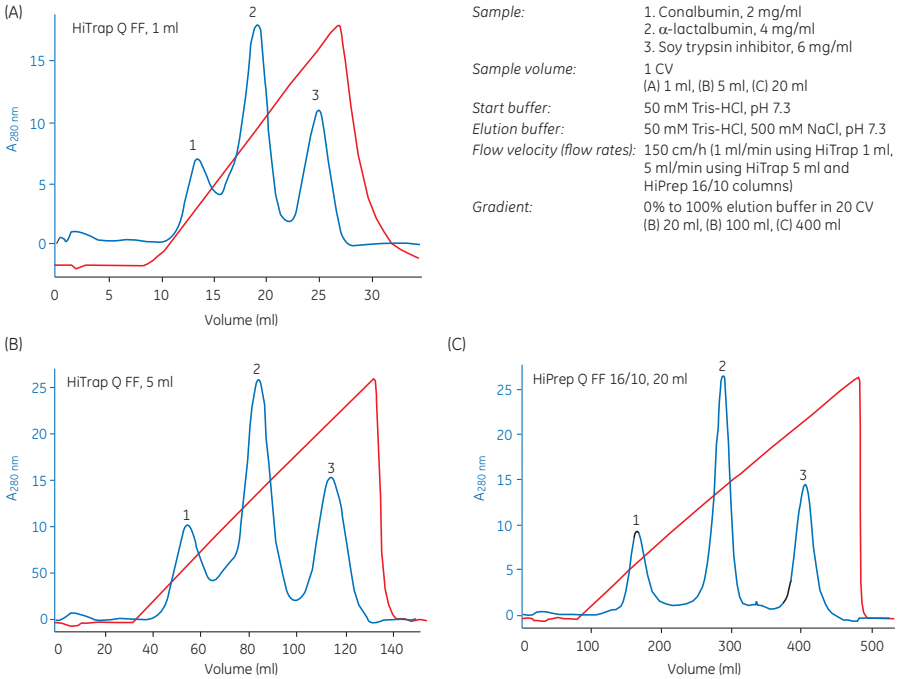


Fig 3.30. Five-fold and 20-fold scale-up using prepacked Q Sepharose Fast Flow prepacked columns.

Sample concentration

It can be an advantage to concentrate a sample prior to SEC in order to minimize sample volume and facilitate a rapid, high-resolution size separation. HiTrap columns offer a convenient, ready-to-use solution for sample concentration. Table 3.11 earlier in the chapter gives examples of the high concentration factors achieved when concentrating proteins from very dilute starting material using HiTrap columns prepacked with Sepharose High Performance medium. Similar results can be achieved with HiTrap columns prepacked with Sepharose Fast Flow or Sepharose XL media.

Performing a separation

Guidelines for selection of media, buffer, pH, and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions using filters of 1 µm or less. To avoid formation of air bubbles in a packed column, maintain buffers and columns at a constant temperature before and during a run.

The pH of the start buffer should be at least 0.5 to 1.0 pH unit above the pI of the target substance when using an anion exchanger (Q, DEAE, or ANX) and 0.5 to 1.0 pH unit below the pI of the target substance when using a cation exchanger (SP, CM). See Appendix 2 for recommendations on volatile and nonvolatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

anion exchange (Q)

start buffer: 20 mM Tris-HCl, pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

cation exchange (SP)

start buffer: 20 mM 2-(N-Morpholino)ethanesulfonic acid (MES), pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

If selectivity is not satisfactory when using a strong ion exchanger (Q or SP), try a weak ion exchanger (DEAE, ANX, or CM) instead.

Users of ÄKTA systems with automatic buffer preparation functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8.0 or cation exchange chromatography at pH 6.0, see *ÄKTA Laboratory-scale Chromatography Systems: Instrument Management Handbook*, 29010831.

First-time use or after long-term storage

1. To remove ethanol, wash with 1 CV of distilled water at 1 ml/min (HiTrap 1 ml, HiScreen 4.7 ml), 5 ml/min (HiTrap 5 ml), 2 ml/min (HiPrep 20 ml), or at 50 cm/h for Sepharose Fast Flow packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 CV of start buffer at 1 ml/min (HiTrap 1 ml), 2.3 ml/min (HiScreen 4.7 ml), 5 ml/min (HiTrap 5 ml), or 5 ml/min (HiPrep 20 ml).
3. Wash with 5 CV of elution buffer, same flow as step 2.
4. Wash with 5 CV of start buffer, same flow as step 2.

Separation by gradient elution

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 2.3 ml/min (HiScreen 4.7 ml), 5 ml/min (HiPrep 20 ml), or at 150 cm/h for Sepharose Fast Flow packed in larger columns. Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10 to 20 CV and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 CV of 1 M NaCl (100%B) to elute any remaining ionically bound material.
6. Re-equilibrate with 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 2.3 ml/min (HiScreen 4.7 ml), 5 ml/min (HiPrep 20 ml), or at 150 cm/h for Sepharose Fast Flow packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable, that is, when all unbound material has washed through the column.
4. Elute the target protein with 5 CV of start buffer containing NaCl at chosen ionic strength.
5. If necessary: repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 CV of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically bound material.
7. Re-equilibrate 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

If ionic detergents have been used, wash the column with 5 CV of distilled water, followed by 2 CV of 2 M NaCl. Re-equilibrate with at least 10 CV of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove nonionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Refer to Chapter 2 for advice on optimizing the separation.

Check column performance regularly by determining column efficiency and peak symmetry, see Appendix 3. Note that Appendix 3 does not apply to HiTrap or HiPrep columns.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

Reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step varies according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this can affect the column packing and interfere with performance.

Removing common contaminants

Flow rates: 1 ml/min (HiTrap 1 ml, HiScreen 4.7 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml), or at 40 cm/h with a contact time of 1 to 2 h for Sepharose Fast Flow packed in larger columns.

1. Wash with at least 2 CV of 2 M NaCl.
2. Wash with at least 4 CV of 1 M NaOH.
3. Wash with at least 2 CV of 2 M NaCl.
4. Rinse with at least 2 CV of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 CV of start buffer or storage buffer until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins, refer to Appendix 1.

Chemical stability

For daily use, Sepharose Fast Flow media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as nonionic detergents, 70% ethanol, 1 M acetic acid, and 30% isopropanol.

Sepharose Fast Flow can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane, and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.

Avoid cationic detergents with SP or CM Sepharose Fast Flow. Avoid anionic detergents with Q, DEAE, or ANX Sepharose Fast Flow. Avoid oxidizing agents.

Storage

For column storage, wash with 2 CV of distilled water followed by 2 CV of 20% ethanol. Include 0.2 M sodium acetate in the 20% ethanol solution for SP Sepharose Fast Flow. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at 4°C to 8°C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at 4°C to 30°C in 20% ethanol. Do not freeze.

To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

Sepharose XL: for selected proteins that require very high binding capacity to increase productivity, easy scale-up

- Use Sepharose XL media for purification of proteins when improved binding capacity compared to other Sepharose media has been confirmed for the selected protein.
- Use Sepharose XL at the beginning of a purification scheme for initial capture when a high binding capacity and rapid separation is required for a selected protein from clarified samples.
- Run columns packed with Sepharose XL on systems such as ÄKTA, HPLC, or systems using peristaltic pumps. Appendix 4 provides guidance on selecting the right ÄKTA system.

Sepharose XL media are based on a matrix of 90 µm particles, made from 6% agarose and highly cross-linked for chemical and physical stability, substituted with quaternary ammonium (Q) or sulfopropyl (SP) groups. The ionic groups are bound to long, flexible dextran chains which have been coupled to the agarose. This increases the exposure of the Q or SP groups thereby raising the binding capacity to a very high level without restricting the passage of charged molecules. The strong ion exchange groups maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application. Particle size and bed volumes remain stable, despite changes in ionic strength or pH, to ensure fast separations at high flow rates with good resolution.

Media characteristics

Composition: sulfopropyl (SP) or quaternary amino (Q) groups attached via chemically stable ether bonds to long, flexible dextran chains that are covalently coupled to highly cross-linked 6% agarose (Table 3.15).

Table 3.15. Characteristics of Sepharose XL media

Product	Functional group	pH stability[1]	Mean particle size (µm)
Q Sepharose XL	$-CH_2-N^+-(CH_3)_3$	Long term: 2 to 12 Short term: 2 to 14	90
SP Sepharose XL	$-CH_2-CH_2-CH_2-SO_3^-$	Long term: 4 to 13 Short term: 3 to 14	90

[1] *Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance. Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. All ranges are estimates based on experience and knowledge gained at GE.*

Purification options

Q and SP Sepharose XL are available in chromatography media packs as well as convenient prepacked HiTrap columns for small-scale purification and HiPrep columns for scale-up (Fig 3.31). Purification options for the media and prepacked columns are shown in Table 3.16.

Fig 3.31. Q and SP Sepharose XL are available in prepacked HiTrap and HiPrep columns, in chromatography media packs, and in the HiTrap IEX Selection Kit, 17600233.

Table 3.16. Purification options for Sepharose XL media and prepacked columns

Product	Binding capacity per column or per ml medium	Recommended working flow[1]	Maximum flow[1]	Working pH range[2]	Maximum operating back pressure[3] (MPa/psi) *1 MPa = 10 bar*
Strong anion exchangers					
Q Sepharose XL	> 130 mg/ml (BSA, M_r 67 000)	300 to 500 cm/h	700 cm/h	2 to 12	0.3/43
HiTrap Q XL, 1 ml	> 130 mg (BSA, M_r 67 000)	up to 1 ml/min	4 ml/min	2 to 12	0.3/43
HiTrap Q XL, 5 ml	> 650 mg (BSA, M_r 67 000)	up to 5 ml/min	20 ml/min	2 to 12	0.3/43
HiPrep Q XL 16/10, 20 ml	> 2600 mg (BSA, M_r 67 000)	2 to 10 ml/min	10 ml/min	2 to 12	0.15/22
Strong cation exchangers					
SP Sepharose XL	> 160 mg/ml (lysozyme, M_r 14 500)	300 to 500 cm/h	700 cm/h	4 to 13	0.3/43
HiTrap SP XL, 1 ml	> 160 mg (lysozyme, M_r 14 500)	up to 1 ml/min	4 ml/min	4 to 13	0.3/43
HiTrap SP XL, 5 ml	> 800 mg (lysozyme, M_r 14 500)	up to 5 ml/min	20 ml/min	4 to 13	0.3/43
HiPrep SP XL 16/10, 20 ml	> 3200 mg (lysozyme, M_r 14 500)	2 to 10 ml/min	10 ml/min	4 to 13	0.15/22

[1] *See Appendix 5 to convert linear flow (cm/h) to volumetric flow rate (ml/min) and vice versa.*
[2] *Working pH range refers to the pH interval where the medium can be operated as intended without adverse long-term effects.*
[3] *Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

Use prepacked HiTrap columns (1 ml or 5 ml) for media selection, method scouting, group separations, small-scale purification, sample concentration, or clean-up. Connect up to three HiTrap columns in series to scale-up.

Use prepacked HiPrep columns (20 ml) for method development, group separations, larger scale purification, sample concentration or clean-up. Connect several HiPrep columns in series to increase binding capacity.

For column packing in Tricorn and XK columns, see Table 3.17. Select a production column such as BPG or Chromaflow for larger volumes.

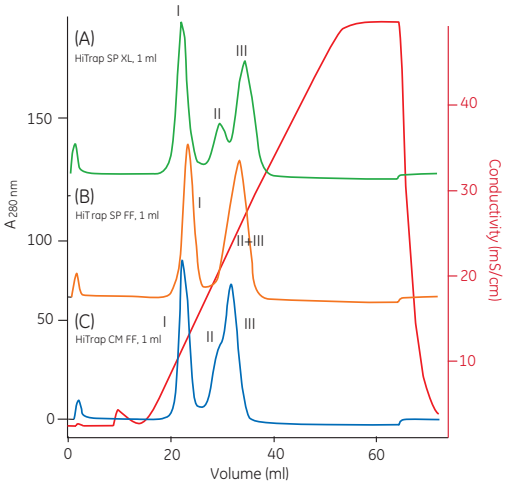
Table 3.17. Packing volumes and bed heights for Sepharose XL media packed in Tricorn and XK columns

Column	Volume (ml)	Bed height (cm)
Tricorn 10/100	up to 8	up to 10
Tricorn 10/150	up to 12	up to 15
Tricorn 10/200	up to 16	up to 20
XK 16/20	up to 30	up to 15
XK 26/20	up to 80	up to 15
XK 26/40	up to 196	> 15
XK 50/20	up to 274	up to 14
XK 50/30	up to 559	up to 28.5

Purification examples

Media selection

The most suitable matrix and charged group for a separation can be selected quickly and easily by using 1 ml HiTrap columns. In Figure 3.32, a comparison of elution profiles for the same sample separated under identical conditions on three different media illustrates the differences in selectivity and resolution that can result from changing the charge group and matrix. The most suitable medium can be selected and conditions optimized according to the requirements for the purification. In this example, Sepharose XL resolves the three components and optimization of elution conditions could further improve the resolution. However, any of these media would be suitable if the aim was to isolate the first major peak (ribonuclease A).

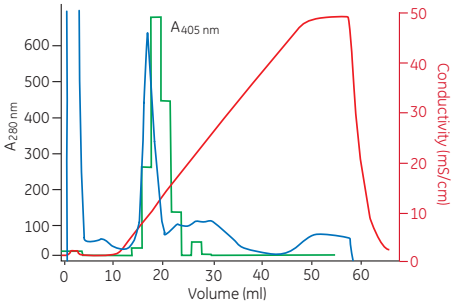


Columns:	(A) HiTrap SP XL, 1 ml (B) HiTrap SP FF, 1 ml (C) HiTrap CM FF, 1 ml
Sample:	3 mg ribonuclease A (pI = 9.3), 0.8 mg cytochrome C (pI = 10.3), 0.8 mg lysozyme (pI > 11)
Sample volume:	2 ml in start buffer
Start buffer:	20 mM sodium phosphate, pH 6.8
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl pH 6.8
Flow rate (flow velocity):	1 ml/min (150 cm/h)
Gradient:	0% elution buffer (25 CV), 0% to 100% elution buffer (40 CV)

Fig 3.32. Media scouting: separation of ribonuclease A (I), cytochrome C (II), and lysozyme (III) on a range of anion exchange HiTrap columns.

Capture

Capture of alkaline phosphatase from a clarified lysate of *E. coli* using a HiTrap Q XL 1 ml column is shown in Figure 3.33. Separation was monitored at A_{280} nm and phosphatase activity assayed by a spectrophotometric method at A_{405} nm.



Columns:	HiTrap Q XL, 1 ml
Sample:	2 ml of *E.coli* lysate clarified by centrifugation
Sample volume:	2 ml
Start buffer:	20 mM Tris-HCl, pH 7.4
Elution buffer:	20 mM Tris-HCl, 500 mM NaCl, pH 7.4
Flow rate:	1 ml/min (150 cm/h)
Gradient:	0% elution buffer (30 CV), 0% to 100% elution buffer (40 CV)

Fig 3.33. Clarified *E. coli* lysate on HiTrap Q XL, absorbance values at 450 nm relate to phosphatase activity in eluted fractions.

Capture and scale-up

Figure 3.34 shows a pilot scale purification performed on a Sepharose XL ion exchanger. The separation was developed on Q Sepharose XL packed in an XK 16/20 column in order to select optimal pH and to determine maximum binding capacity available. Adding $CaCl_2$ to the sample precipitated DNA and so increased the binding capacity for the target protein. Final loading was reduced to 75% of the maximum capacity and the result verified before scaling-up to a larger, INdex column.

Column:	Q Sepharose XL in INdEX 70 column, 385 ml bed volume
Sample:	Recombinant α-amylase produced in *E. coli*, homogenized, 2.2 l diluted in distilled water to 15.4 l, 7.2 mS/cm, 10 mM $CaCl_2$, centrifuged
Start buffer:	20 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$
Elution buffer:	20 mM Tris-HCl, pH 8.0, 1 M NaCl, 10 mM $CaCl_2$
Flow flow:	300 cm/h, 12 l/h
Gradient:	20 CV 0 to 1 M NaCl
Eluent:	1.48 l, 3.8 CV

Spec. act. α-amylase 6420 U/l

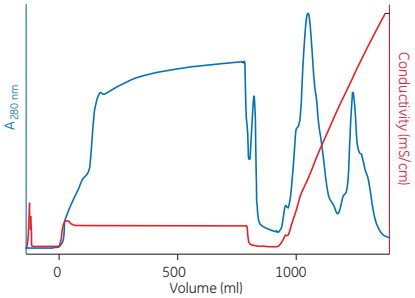


Fig 3.34. Capture of recombinant α-amylase from *E. coli* on Q Sepharose XL pilot scale column. The first peak during gradient elution contained **α**-amylase.

Sample concentration

It can be an advantage to concentrate a sample prior to SEC in order to minimize sample volume and facilitate a rapid, high resolution size separation. HiTrap columns offer a convenient, ready-to-use solution for sample concentration. Table 3.9 earlier in the chapter gives examples of the high concentration factors achieved when concentrating proteins from very dilute starting material using HiTrap columns prepacked with Sepharose High Performance medium. Similar results can be achieved with HiTrap columns prepacked with Sepharose Fast Flow or Sepharose XL media.

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions, and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions using filters of 1 µm or smaller. To avoid formation of air bubbles in a packed column, maintain buffers and columns at a constant temperature before and during a run.

The pH of the start buffer should be at least 0.5 to 1.0 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5 to 1.0 pH unit below the pI of the target substance when using a cation exchanger (SP). See Appendix 2 for recommendations on volatile and nonvolatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

anion exchange (Q)
start buffer: 20 mM Tris-HCl, pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

cation exchange (SP)
start buffer: 20 mM 2-(N-Morpholino)ethanesulfonic acid (MES), pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTA systems with automatic buffer preparation functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8.0 or cation exchange chromatography at pH 6.0, see *ÄKTA Laboratory-scale Chromatography Systems: Instrument Management Handbook*, 29010831.

First-time use or after long-term storage

1. To remove ethanol, wash with 1 CV of distilled water at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml), or at 50 cm/h for Sepharose XL packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 CV of start buffer at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml), or at 150 cm/h for Sepharose XL packed in larger columns.
3. Wash with 5 CV of elution buffer, same flow as step 2.
4. Wash with 5 CV of start buffer, same flow as step 2.
5. Run a blank elution before applying sample.

Separation by gradient elution

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml), or at 150 cm/h for Sepharose XL packed in larger columns. Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable, that is, when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10 to 20 CV and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 CV of 1 M NaCl (100%B) to elute any remaining ionically bound material.
6. Re-equilibrate 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml), or at 150 cm/h for Sepharose XL packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable, that is, when all unbound material has washed through the column.
4. Elute the target protein with 5 CV of start buffer containing NaCl at chosen ionic strength.
5. If necessary: repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 CV of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically bound material.
7. Re-equilibrate with 5 to 10 CV of start buffer or until eluent pH and conductivity reach the required values.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

If ionic detergents have been used, wash the column with 5 CV of distilled water, followed by 2 CV of 2 M NaCl. Re-equilibrate with at least 10 CV of start buffer until the UV baseline, eluent pH and/or conductivity are stable.

Organic solvents such as ethanol can be used to remove nonionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure, or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

Reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step varies according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this can affect the column packing and interfere with performance.

Removing common contaminants

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml), or at 40 cm/h with a contact time of 1 to 2 h for Sepharose XL packed in larger columns.

1. Wash with at least 2 CV of 2 M NaCl.
2. Wash with at least 4 CV of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 CV of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 CV of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 CV of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins, refer to Appendix 1.

Chemical stability

For daily use, Sepharose XL media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as nonionic detergents, 70% ethanol, 1 M acetic acid, and 30% isopropanol.

Sepharose XL can be used with organic solvents such as dimethylsulfoxide, dimethyl-formamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane, and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.

Avoid cationic detergents with SP Sepharose XL. Avoid anionic detergents with Q Sepharose XL. Avoid oxidizing agents.

Storage

For column storage, wash with 2 CV of distilled water followed by 2 CV of 20% ethanol. Include 0.2 M sodium acetate in the storage solution for SP Sepharose XL. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid overpressuring the column. Store at room temperature or, for long periods, store at 4°C to 8°C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at 4°C to 30°C in 20% ethanol. Do not freeze.

To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

Sepharose Big Beads: purification from crude, viscous samples at large scale

- Use Sepharose Big Beads for purification of proteins from crude, viscous samples.
- Use Sepharose Big Beads when handling large volumes of crude or viscous samples that must be bound rapidly and when resolution is less important.
- Use Sepharose Big Beads for capture steps, when viscosity and back pressure can limit the throughput attainable with ion exchangers of smaller particle size.
- Run columns packed with Sepharose Big Beads on systems such as ÄKTA, HPLC, or systems using peristaltic pumps. Appendix 4 gives guidance on how to select the most suitable ÄKTA system.

Sepharose Big Beads are ion exchangers designed for large-scale industrial applications. Sepharose Big Beads are based on 100 to 300 µm, cross-linked 6% agarose particles, substituted with quaternary ammonium (Q) or sulfopropyl (SP) groups. The large particle size, together with a high degree of cross-linking for extreme physical and chemical stability, ensures that high flow rates can be maintained when processing very viscous samples. For example, a flow of 500 cm/h can be maintained in an industrial process at viscosities up to 2.5 times the viscosity of water. More dilute samples can be run at 1000 cm/h. Particle size and bed volumes remain stable, despite changes in ionic strength or pH. The strong ion exchange groups (Q and SP) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Figures 3.35 and 3.36 show the excellent flow characteristics and typical binding capacities for Sepharose Big Beads media.

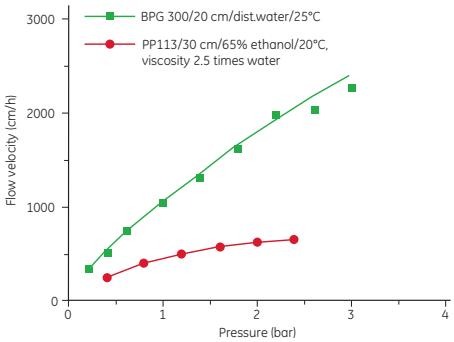
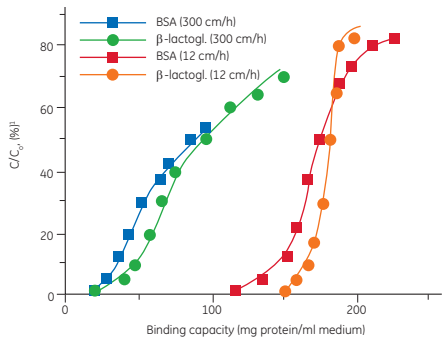


Fig 3.35. Sepharose Big Beads allow high flow rates with high-viscosity samples.



[1] *Concentration in eluate (C) as fraction of concentration in sample (Co).*

Fig 3.36. Typical binding capacities of SP Sepharose Big Beads. Binding capacity measured in acetate pH 5.0 for bovine serum albumin (BSA) and formate pH 4.1 for β-lactoglobulin at flow velocities of 12 and 300 cm/h.

Media characteristics

Composition: sulfopropyl (SP) or quaternary amino (Q) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds, see Table 3.18.

Table 3.18. Characteristics of Sepharose Big Beads

Product	Functional group	pH stability[1]	Mean particle size (µm)
Strong anion exchanger			
Q Sepharose Big Beads	$-CH_2-N^+-(CH_3)_3$	Long term: 2 to 12 Short term: 2 to 14	200
Strong cation exchanger			
SP Sepharose Big Beads	$-CH_2-CH_2-CH_2-SO_3^-$	Long term: 4 to 13 Short term: 3 to 14	200

[1] *Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance. Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. All ranges are estimates based on experience and knowledge gained at GE.*

Purification options

Purification options for Q and SP Sepharose Big Beads are described in Table 3.19.

Table 3.19. Purification options for Sepharose Big Beads

Product	Binding capacity per column or per ml medium	Recommended working flow[1]	Maximum flow[1]	Working pH range[2]	Maximum operating back pressure[3] (MPa/psi) *1 MPa = 10 bar*
Strong anion exchanger					
Q Sepharose Big Beads	Tested for each specific application	up to 300 cm/h	1800 cm/h	2 to 12	0.3/43
Strong cation exchanger					
SP Sepharose Big Beads	Tested for each specific application	up to 300 cm/h	1800 cm/h	4 to 13	0.3/43

[1] *See Appendix 5 to convert flow velocity (cm/h) to volumetric flow rate (ml/min) and vice versa.*
[2] *Working pH range refers to the pH interval where the medium can be operated as intended without adverse long-term effects.*
[3] *Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

For column packing in XK columns during method development, particularly when handling crude, viscous samples, see Table 3.20.

Table 3.20. Packing volumes and bed heights for Sepharose Q and SP High Performance packed in XK columns

Column	Volume (ml)	Bed height (cm)
XK 16/20	up to 30	up to 15
XK 26/20	up to 80	up to 15
XK 26/40	up to 196	> 15
XK 50/20	up to 274	up to 14
XK 50/30	up to 559	up to 28.5

Select a production scale column such as BPG or Chromaflow for larger volumes. Sepharose

Big Beads can be packed in large scale columns by applying constant pressure between 0.1 to 0.3 MPa (1.0 to 3.0 bar, 14.5 to 43.5 psi) by slurry sedimentation followed by adapter compression, or by suction packing. Follow the instructions supplied with the medium.

Performing a separation

Guidelines for selection of media, buffer, pH, and ionic strength conditions, and method optimization are given in Chapter 2. See Appendix 2 for recommendations on volatile and nonvolatile buffer systems.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Refer to Chapter 2 and Appendix 1 for recommendations and advice.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 1 µm filters. To avoid formation of air bubbles in a packed column, maintain buffers and columns at a constant temperature before and during a run.

First-time use or after long-term storage

1. Wash with 5 CV of distilled water at 300 cm/h.
2. Wash with 5 CV of start buffer, same flow as step 1.
3. Wash with 5 CV of elution buffer, same flow as step 1.
4. Wash with 5 CV of start buffer, same flow as step 1.

Gradient or step elution

Conditions for a large scale-purification using Sepharose Big Beads will be determined during method development and relate to the specific application. Refer to Chapter 2 for advice on optimizing a separation. Typical separation flow rates should be 200 to 500 cm/h.

If ionic detergents have been used, wash the column with 5 CV of distilled water, followed by 2 CV of 2 M NaCl. Re-equilibrate with at least 10 CV of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove nonionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

Cleaning

Correct preparation of samples and buffers and regeneration with a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure, or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

Reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step varies according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this can affect the column packing and interfere with performance.

Removing common contaminants

1. Wash with at least 2 CV of 2 M NaCl at 40 cm/h for a contact time of 1 to 2 h.
2. Wash with at least 4 CV of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 CV of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 CV of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 CV of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins, refer to Appendix 1.

Chemical stability

For daily use, Sepharose Big Beads are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as nonionic detergents, 70% ethanol, 1 M acetic acid, 30% acetonitrile, and 30% isopropanol.

Sepharose Big Beads can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50), as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.

Avoid cationic detergents with SP Sepharose Big Beads. Avoid anionic detergents with Q Sepharose Big Beads. Avoid oxidizing agents.

Storage

For column storage, wash with 2 CV of distilled water followed by 2 CV of 20% ethanol. Include 200 mM sodium acetate in the storage solution for SP Sepharose Big Beads. For small-scale columns, degas the ethanol/water mixture thoroughly, for large-scale columns ensure that an air trap is included before the column. Add storage solution at a low flow rate, checking the back pressure as the column equilibrates. Alternatively, store at neutral pH in buffer containing 20% ethanol or in 100 mM NaOH.

Store at room temperature or, for long periods, store at 4°C to 8°C. Ensure that the column is sealed well to avoid drying out. Store unused media at 4°C to 30°C in 20% ethanol. Do not freeze.

Capto: high-flow media with high resolution

- Use Capto media, including Capto, Capto ImpRes and Capto ImpAct, for screening of selectivity and method conditions before scaling up, as well as for small-scale purification.
- Use Capto media with high flow-properties for capture, intermediate purification, and polishing of a wide range of biomolecules.
- Run Capto media in an optimal way with liquid chromatography systems such as ÄKTA . Appendix 4 provides guidance on selecting the right ÄKTA system.
- Capto media are based on the high-flow agarose base matrix, which gives excellent pressure-flow properties, making the media suitable for process- scale applications (Fig 3.37).

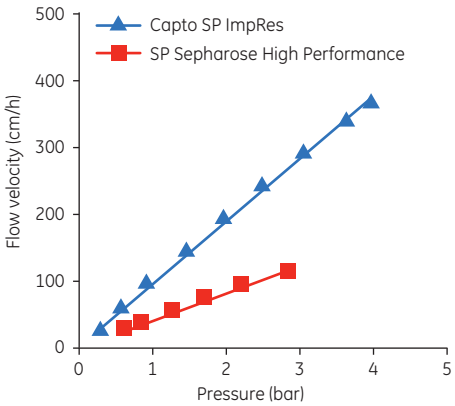


Fig 3.37. The pressure-flow properties of Capto ImpRes are improved compared with Sepharose High Performance due to the increased mechanical stability of the base matrix. Running conditions: Axichrom™ 300 column, 20 cm bed height with water at 20°C.

Capto media are high capacity ion exchangers where the ligands are coupled to a chemically modified, high-flow agarose matrix. The high-flow agarose matrix provides particle rigidity without compromising the pore size. This allows for fast mass transfer resulting in high dynamic binding capacities at high flow rates, making the media suitable for process-scale applications.

Capto Q, Capto S, and Capto DEAE are used for capture and intermediate purification of proteins. Capto Q ImpRes and Capto SP ImpRes are high-resolution media designed for intermediate purification and polishing. Capto S ImpAct is especially suitable for intermediate purification and polishing of MAbs, where a common main challenge is to selectively remove impurities similar to the target product.

Media characteristics

Composition: Quaternary amine (Q), sulfopropyl (SP), and sulfonate (S) coupled to highly cross-linked high-flow agarose. The characteristics of Capto media are shown in Table 3.21.

Table 3.21. Characteristics Capto media

Product	Functional group	pH stability[1]	Mean particle size (µm)
Capto Q	$-CH_2-N^+-(CH_3)_3$	Long term: 2 to 12 Short term: 2 to 14	90
Capto DEAE	$-CH_2-CH_2-N^+-(CH_2CH_3)_2$	Long term: 2 to 12 Short term: 2 to 14	90
Capto S	$-CH_2-SO_3^-$	Long term: 4 to 12 Short term: 3 to 14	90
Capto Q ImpRes	$-CH_2-N^+-(CH_3)_3$	Long term: 2 to 12 Short term: 2 to 14	40
Capto SP ImpRes	$-CH_2-CH_2-CH_2-SO_3^-$	Long term: 4 to 12 Short term: 3 to 14	40
Capto S ImpAct	$-CH_2-SO_3^-$	Long term: 4 to 12 Short term: 3 to 14	50

[1] *Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance. Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place, and sanitization procedures. All ranges are estimates based on experience and knowledge gained at GE.*

Purification options

Anion and cation exchanger Capto media are available prepacked in HiTrap and HiScreen columns and in chromatography media packs (Fig 3.38). Table 3.22 is a presentation of different Capto media for ion exchange chromatography. Some of the media are also available in PreDictor™ filter plates, PreDictor RoboColumn™, and ReadyToProcess™ columns (see Chapter 5, *Large-scale purification*).

Fig. 3.38. Capto media are available in bulk packs, and prepacked HiTrap and HiScreen columns, The media are also available in high-throughput process development (HTPD) format.

Table 3.22. Purification options for Capto media

	Binding capacity	Recommended working flow rate	Maximum flow rate	Working pH range
Strong anion exchangers				
Capto Q	> 100 mg (BSA)/ml medium	up to 700 cm/h[1]	700 cm/h[1]	2 to 12
Capto Q ImpRes	> 55 mg (BSA)/ml medium	up to 220 cm/h[1]	220 cm/h[1]	2 to 12
HiTrap Capto Q, 1 ml	> 100 mg (BSA)	1.0 ml/min	4.0 ml/min	2 to 12
HiTrap Capto Q, 5 ml	> 500 mg (BSA)	5.0 ml/min	20.0 ml/min	2 to 12
HiTrap Capto Q ImpRes, 1 ml	> 55 mg (BSA)	1.0 ml/min	4.0 ml/min	2 to 12
HiTrap Capto Q ImpRes, 5 ml	> 275 mg (BSA)	5.0 ml/min	20.0 ml/min	2 to 12
HiScreen Capto Q, 4.7 ml	> 470 mg (BSA)	1.2 ml/min	2.3 ml/min	2 to 12
HiScreen Capto Q ImpRes, 4.7 ml	> 260 mg (BSA)	1.2 ml/min	2.3 ml/min	2 to 12
Weak anion exchangers				
Capto DEAE	> 90 mg (ovalbumin)/ml medium	up to 700 cm/h[1]	700 cm/h[1]	2 to 12
HiTrap Capto DEAE, 1 ml	> 90 mg (ovalbumin)	1.0 ml/min	4.0 ml/min	2 to 12
HiTrap Capto DEAE, 5 ml	> 450 mg (ovalbumin)	5.0 ml/min	20.0 ml/min	2 to 12
HiScreen Capto DEAE, 4.7 ml	> 420 mg (ovalbumin)	1.2 ml/min	2.3 ml/min	2 to 12
Strong cation exchangers				
Capto S	> 120 mg (lysozyme)/ml medium	up to 700 cm/h[1]	700 cm/h[1]	4 to 12
Capto SP ImpRes	> 70 mg (lysozyme)/ml medium	up to 220 cm/h[1]	220 cm/h[1]	4 to 12
Capto S ImpAct	> 90 mg (lysozyme)/ml medium	up to 220 cm/h[1]	220 cm/h[1]	4 to 12
HiTrap Capto S, 1 ml	> 120 mg (lysozyme)	1.0 ml/min	4.0 ml/min	4 to 12
HiTrap Capto S, 5 ml	> 600 mg (lysozyme)	5.0 ml/min	20.0 ml/min	4 to 12
HiTrap Capto SP ImpRes, 1 ml	> 70 mg (lysozyme)	1.0 ml/min	4.0 ml/min	4 to 12
HiTrap Capto SP ImpRes, 5 ml	> 350 mg (lysozyme)	5.0 ml/min	20.0 ml/min	4 to 12
HiTrap Capto S ImpAct, 1 ml	> 90 mg (lysozyme)	1.0 ml/min	4.0 ml/min	4 to 12
HiTrap Capto S ImpAct, 5 ml	> 450 mg (lysozyme)	5.0 ml/min	20.0 ml/min	4 to 12
HiScreen Capto S, 4.7 ml	> 560 mg (lysozyme)	1.2 ml/min	2.3 ml/min	4 to 12
HiScreen Capto SP ImpRes, 4.7 ml	> 330 mg (lysozyme)	1.2 ml/min	2.3 ml/min	4 to 12
HiScreen Capto S ImpAct, 4.7 ml	> 420 mg (lysozyme)	1.2 ml/min	2.3 ml/min	4 to 12

[1] *Flow velocity in a 1 m column with 20 cm bed height at 20°C using process buffers with the same viscosity as water at < 0.3 MPa (3 bar, 43.5 psi). See Appendix 5 to convert flow velocities (cm/h) to volumetric flow rate (ml/min) and vice versa.*

[2] *Working pH range refers to the pH interval where the medium can be operated as intended without adverse long-term effects. Molecular weights (M_r) of proteins in the table: BSA, 67 000; ovalbumin, 45 000; lysozyme, 14 500.*

- Use prepacked HiTrap columns (1 ml or 5 ml) for media selection, group separations, and small-scale purification.
- Use prepacked HiScreen columns (4.7 ml, 10 cm bed height) for method development and optimization before scaling up.
- For column packing: Capto media can be used with most modern chromatography equipment from laboratory to production scale. Due to the higher rigidity of Capto media, packing procedures differ slightly compared with Sepharose (for details of packing laboratory-scale columns, see the appropriate Instructions).

Table 3.23 lists suitable empty columns for packing of Capto media from GE.

Table 3.23. Example of columns suitable for packing of Capto media

Column family	Inner diameter (mm)
Laboratory scale	
Tricorn	5, 10
HiScale™	16, 26, 50
Pilot and production scale	
AxiChrom	50 to 1000[1]
BPG	100 to 300[2]

[1] *Maximum bed height for AxiChrom 1000 is 20 cm.*
[2] *Note that the pressure rating of BPG 450 column is too low for Capto ImpRes media.*

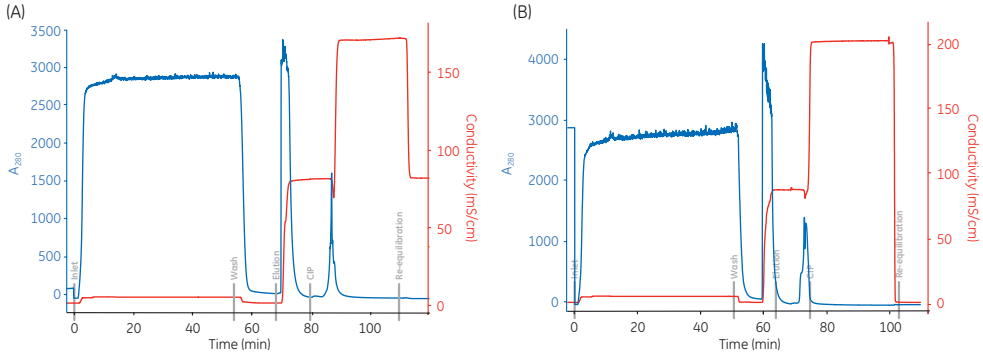
Purification examples

Capture and scale-up

Capto media belong to the BioProcess™ range of media that are developed and supported for production-scale chromatography. The small prepacked column formats, HiTrap 1 ml and 5 ml and HiScreen (4.7 ml), are convenient to use together with a chromatography system when developing efficient and robust separation methods. Further development and optimization using Tricorn or HiScale columns then permits straightforward scale-up. Figure 3.39 shows an example of scaling up an optimized purification of α-chymotrypsin on Capto S, starting from a Tricorn column.

Column:	Tricorn 5/100 (bed height 9.7 cm, CV = 1.9 ml)
Medium:	Capto S
Sample:	α-chymotrypsin in *E. coli* homogenate, 4 mg/ml to 50 ml
Start buffer:	50 mM sodium acetate, pH 4.8
Elution buffer:	50 mM sodium acetate, 1 M NaCl, pH 4.8
Flow velocity:	285 cm/h
Gradient:	0% to 100% 0 CV, 100% 5 CV
Residence time:	2 min

Column:	XK 16/40 (bed height 20.7 cm, CV = 41.5 ml)
Medium:	Capto S
Sample:	α-chymotrypsin in *E. coli* homogenate, 4 mg/ml to 1040 ml
Start buffer:	50 mM sodium acetate, pH 4.8
Elution buffer:	50 mM sodium acetate, 1 M NaCl, pH 4.8
Flow velocity:	624 cm/h
Gradient:	0% to 100% 0 CV, 100% 5 CV
Residence time:	2 min



Column:	AxiChrom 50 (bed height 22 cm, CV = 431 ml)
Medium:	Capto S
Sample:	α-chymotrypsin in *E. coli* homogenate, 4 mg/ml to 10.8 l
Start buffer:	50 mM sodium acetate, pH 4.8
Elution buffer:	50 mM sodium acetate, 1 M NaCl, pH 4.8
Flow velocity:	645 cm/h
Gradient:	0% to 100% 0 CV, 100% 5 CV
Residence time:	2 min

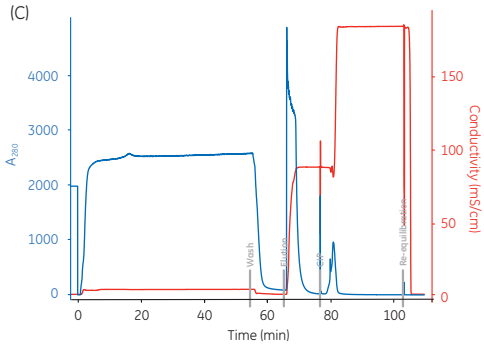


Fig 3.39. A 200-fold scale up of α-chymotrypsin on Capto S packed in (A) Tricorn 5/100, (B) XK 16/40 and, (C) AxiChrom columns.

Intermediate purification of insulin

Capto SP ImpRes was used for intermediate purification where the purpose was to separate insulin from components remaining from the initial capture step. The resulting chromatogram is shown in Figure 3.40. Insulin (first peak) was well-resolved from truncated insulin impurities (middle peak) and C-peptides (third peak). Pooled fractions were analyzed by RPC, which showed an increase of insulin purity from 64% to 91%. The truncated insulin content was reduced from 11.5% to 2.8%.

Column:	Tricorn 5/50 CV 1 ml
Medium:	Capto SP ImpRes
Sample:	18 mg cleaved insulin*
Start buffer:	50 mM acetate, 47.5% ethanol, pH 4
Elution buffer:	50 mM acetate, 47.5% ethanol, 1 M NaCl, pH 4
Elution:	First step: 47.5% ethanol, 130 mM NaCl, pH 4 (10 CV) Second step: 47.5% ethanol, 1 M NaCl, pH 4 (5 CV)
Flow rate (flow velocity):	0.4 ml/min (120 cm/h)
Residence time:	2.5 min

* *Kindly provided by Biomm S.A. (Brazil).*

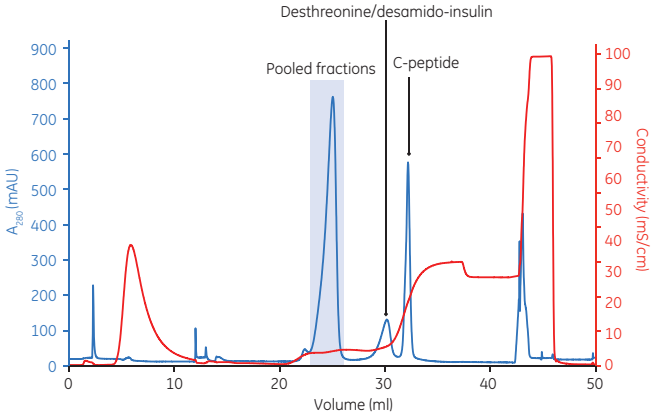


Fig 3.40. The intermediate step purification of insulin on Capto SP ImpRes removes contaminants such as desthreonine- and desamido-insulin.

Polishing of MAb

To evaluate Capto S ImpAct for removal of aggregates, four different MAb purified on MabSelect SuRe™ medium were run using linear gradient elution in Tricorn columns. Fractions from the elution peaks were collected and analyzed by analytical SEC for aggregate content. HCP and protein A content were analyzed using a Gyrolab™ workstation and a commercial ELISA assay, respectively. As can be seen in Table 3.24, Capto S ImpAct demonstrates effective aggregate and HCP removal at a high monomer recovery. Figure 3.45 shows the chromatogram for MAb E, illustrating that aggregates (green) elute at the tail of the elution peak. For this MAb, the aggregate level was reduced to 0.6% at 90% monomer recovery. The initial aggregate content was 2%.

Table 3.24. Results from the purification of four MAb using Capto S ImpAct

	MAb A	MAb C	MAb D	MAb E
DBC (mg/ml medium)	108	92	118	122
Load (mg/ml medium)	80	64	80	85
Start aggregate content (%)	7	2	4	2
Aggregates at 90% monomer recovery (%)	0.9	0.6	1.2	0.6
Start HCP content (ppm)	34	1800	300	454
HCP at 90% monomer recovery (ppm)	8	42	25	43
Start protein A content (ppm)	4	1	1	< 1
Protein A at 90% monomer recovery (ppm)	< 1	< 1	< 1	< 1
Elution pool volume (CV)	5.1	5.4	4.0	6.3
Elution pool concentration at 90% monomer recovery (mg/ml)	13.6	10.6	17.8	12.3

Medium:	Capto S ImpAct
Column:	Tricorn 5/100, bed height 10 cm
Sample:	MAb E, purified on MabSelect SuRe medium
Sample load:	85 mg/ml medium
Start buffer:	50 mM sodium acetate, pH 5.3
Elution buffer:	50 mM sodium acetate + 500 mM NaCl, pH 5.3
Flow rate:	0.35 ml/min, residence time 5.4 min
Gradient:	Linear, 0 to 350 mM NaCl in 20 CV

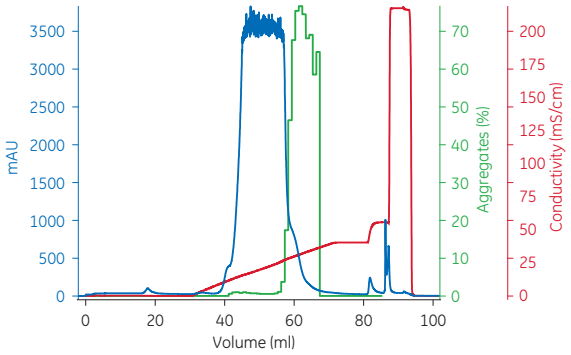


Fig 3.41. Chromatogram from purification of MAb E using Capto S ImpAct in a Tricorn 5/100 column. Histogram in green represents aggregates in fractions. SEC was used for aggregate content analysis, using a prepacked Superdex™ 200 Increase 10/300 GL column.

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers after all salts and additives have been included. Use high quality water and chemicals. Filter solutions using filters of 1 µm or less. To avoid formation of air bubbles in a packed column, maintain buffers and columns at constant temperature before and during a run.

The pH of the start buffer should be at least 0.5 to 1.0 pH unit above the pI of the target substance when using an anion exchanger (Q, DEAE) and 0.5 to 1.0 pH unit below the pI of the target substance when using a cation exchanger (S, SP).

For samples with unknown charge properties, try the following:

Anion exchange (Q)

Start buffer: 20 mM Tris-HCl, pH 8.0
Elution buffer: 20 mM Tris-HCl, 1 M NaCl, pH 8.0

Cation exchange (SP)

Start buffer: 50 mM sodium acetate, pH 5.0
Elution buffer: 50 mM sodium acetate, 1 M NaCl, pH 5.0
or
Start buffer: 50 mM 2-(N-Morpholino)ethanesulfonic acid (MES), pH 6.0
Elution buffer: 50 mM MES, 1 M NaCl, pH 6.0

Users of ÄKTA systems with automatic buffer preparation functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8.0 or cation exchange chromatography at pH 6.0, see *ÄKTA Laboratory-scale Chromatography Systems: Instrument Management Handbook*, 29010831.

First-time use or after long-term storage

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 1.2 ml/min (HiScreen 4.7 ml).

1. Remove the stopper and connect the column to the system (or syringe) with a drop-to-drop connection to avoid introducing air into the column.
2. Remove the snap-off end at the column outlet and wash with 1 column volume (CV) of distilled water. This step ensures removal of ethanol and avoids the precipitation of buffer salts upon exposure to ethanol. The step can be omitted if precipitation is not likely to be a problem.
3. Wash with 5 CV of start buffer.
4. Wash with 5 CV of elution buffer.
5. Wash with 5 CV of start buffer.

Separation by gradient elution

Linear ionic strength gradients should always be used for method development or when starting with an unknown sample. Linear ionic strength gradients are easy to prepare and very reproducible when generated by a suitable chromatography system. The results obtained can then serve as a base from which to optimize the separation.

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 1.2 ml/min (HiScreen 4.7 ml).
Collect fractions throughout the separation.

1. Equilibrate the column with 5 to 10 CV of start buffer or until the UV baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and conductivity and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until no material appears in the effluent.
4. Begin elution using a gradient volume of 10 to 20 CV and an increasing salt concentration up to 500 mM NaCl (50% elution buffer).
5. Wash with 5 CV of 1 M NaCl (100% elution buffer) to elute any remaining ionically bound material.
6. Re-equilibrate with 5 to 10 CV of start buffer or until the UV baseline, eluent pH, and conductivity reach the required values.

Separation by step elution

Reduce separation time and buffer consumption by transferring to a step elution.

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 1.2 ml/min (HiScreen 4.7 ml).
Collect fractions throughout the separation.

1. Equilibrate the column with 5 to 10 CV of start buffer until the UV baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to the chosen starting pH and conductivity and apply to the column.
3. Wash with 5 to 10 CV of start buffer or until no material appears in the effluent.
4. Elute the target protein with 5 CV of start buffer including NaCl at chosen concentration.
5. If necessary: repeat step 4 at higher NaCl concentrations until the target protein has been eluted.
6. Wash with 5 CV of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically bound material.
7. Re-equilibrate with 5 to 10 CV of start buffer or until the UV baseline, eluent pH, and conductivity reach the required values.

Save time by using higher flow rates during the high salt wash and re-equilibration steps.

Do not exceed the maximum recommended flow and back pressure for the column.

Cleaning

Correct preparation of samples and buffers, including a high salt wash (1 to 2 M NaCl) after each purification, should maintain columns in good condition. However, reduced performance, increased back pressure or blockage indicates that the medium needs cleaning.

Removing common contaminants

Flow rates: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 1.2 ml/min (HiScreen 4.7 ml).

1. Wash with at least 2 CV of 2 M NaCl.
2. Wash with at least 4 CV of 1 M NaOH.
3. Wash with at least 2 CV of 2 M NaCl.
4. Rinse with at least 2 CV of distilled water.
5. Wash with at least 5 CV of start buffer until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins, refer to Appendix 1.

Chemical stability

For daily use, Capto media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), 70% ethanol, 30% acetonitrile, and with additives such as nonionic detergents.

Avoid cationic detergents with Capto S, Capto SP ImpRes, and Capto S ImpAct.

Avoid anionic detergents with Capto Q and Capto Q ImpRes.

Avoid oxidizing agents.

Storage

Wash with 2 CV of distilled water followed by 2 CV of 20% ethanol (Capto Q, Capto DEAE, Capto Q ImpRes) or 20% ethanol containing 200 mM sodium acetate (Capto S, Capto SP ImpRes, Capto S ImpAct). Store at 4°C to 30°C. Do not freeze. Ensure that the column is sealed well to avoid drying out.

Capto media with multimodal functionality

Some Capto media have ligands with multimodal functionality, where the ligand interacts with the target protein through two or more modes of action. This multimodal functionality gives a different selectivity compared to standard ion exchangers, which allows multimodal ion exchangers a wider window of operation in circumstances where traditional media are not as effective as desired. Such circumstances can be encountered, for example, when the loading conductivity of the sample is too high for traditional IEX media, when there is a need to reduce the number of purification steps, or when the selectivity of traditional media is insufficient to provide the required purity of the target protein. See the *Multimodal Chromatography Handbook*, 29054808 for more information about our multimodal Capto media.

Chapter 4
Ion exchange in a Purification Strategy (CIPP)

To ensure efficient, reproducible purification giving the required degree of purity, it is beneficial to develop a multistep process using the purification strategy of Capture, Intermediate Purification, and Polishing (CIPP), shown in Figure 4.1.

CIPP is used in both the pharmaceutical industry and in the research laboratory to ensure faster method development, a shorter time to pure product and good economy. This chapter gives a brief overview of this approach which is recommended for any multi-step protein purification. The *Strategies for Purification Handbook* from GE is a definitive guide for planning efficient and effective protein purification strategies. An important first step for any purification is correct sample preparation and this is covered in more detail in Appendix 1 and Chapter 2.

IEX plays a significant and highly flexible role in most multistep purification schemes. If a specific affinity medium is not available or if little is known about the target molecule, IEX is recommended as the first step to consider for any purification. The technique can be used for capture, intermediate purification, or polishing, according to the demands of the specific application. Since IEX offers different selectivities (using anion or cation exchangers) and since the pH of the purification can be modified to alter the charge characteristics of the sample components, it is possible to use the technique more than once in the same purification scheme. In addition, IEX can be used with step elution for a rapid capture step or with gradient elution to achieve the highest resolution in a polishing step.

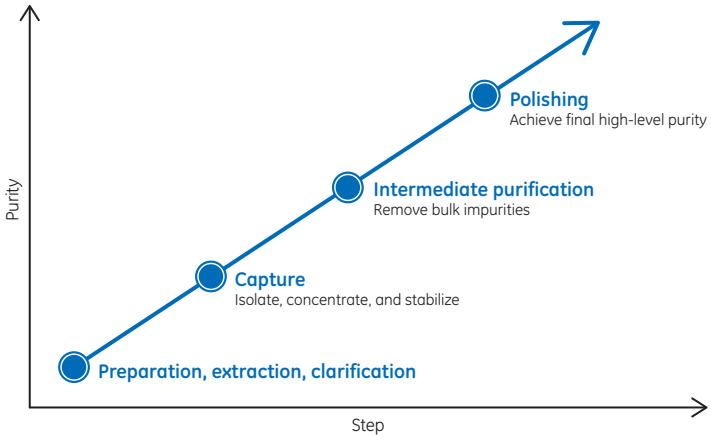


Fig 4.1. Preparation and CIPP.

Applying CIPP

Imagine the purification has three phases: Capture, Intermediate Purification, and Polishing.

Assign a specific objective to each step within the purification process.

The issues associated with a particular purification step will depend greatly upon the properties of the starting material. Thus, the objective of a purification step will vary according to its position in the process.

In the *capture phase*, the objectives are to *isolate, concentrate, and stabilize* the target product. The product should be concentrated and transferred to an environment that will conserve potency/activity.

During the *intermediate purification phase*, the objectives are to *remove most of the bulk impurities*, such as other proteins and nucleic acids, endotoxins, and viruses.

In the *polishing phase*, most impurities have already been removed. The objective is to *achieve final purity* by removing any remaining trace impurities or closely related substances.

The optimal selection and combination of purification techniques for *Capture, Intermediate Purification, and Polishing* is crucial for an efficient purification.

CIPP does not mean that there must always be three purification steps. For example, capture and intermediate purification might be achievable in a single step, as might intermediate purification and polishing. Similarly, purity demands can be so low that a rapid capture step is sufficient to achieve the desired result. For purification of therapeutic proteins, a fourth or fifth purification step might be required to fulfill the highest purity and safety demands. The number of steps used will always depend upon the purity requirements and intended use of the protein.

Selection and combination of purification techniques

Proteins are purified using purification techniques that separate according to differences in specific properties, as shown in Table 4.1.

Table 4.1. Protein properties used during purification

Protein property	Technique
Size	Size exclusion chromatography (SEC)
Charge	Ion exchange chromatography (IEX)
Hydrophobicity	Hydrophobic interaction chromatography (HIC), Reversed phase chromatography (RPC)
Biorecognition (ligand specificity)	Affinity chromatography (AC)

There are four important performance parameters to consider when planning each purification step: resolution, capacity, speed, and recovery. Optimization of any one of these four parameters can be achieved only at the expense of the others, and each purification step will be a compromise (Fig. 4.2). The importance of each parameter will vary depending on whether a purification step is used for capture, intermediate purification, or polishing. Purification methods should be selected and optimized to meet the objectives for each purification step.

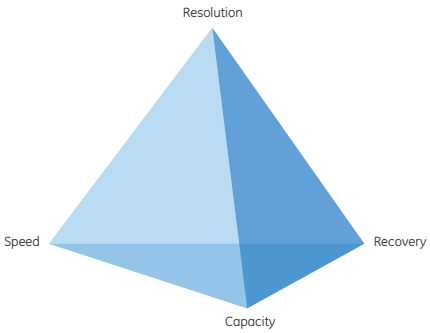


Fig 4.2. Key performance parameters for protein purification. Each purification step should be optimized for one or two of the parameters.

Capacity, in the simple model shown, refers to the amount of target protein loaded during purification. In some cases the amount of sample that can be loaded will be limited by volume (as in SEC) or by large amounts of contaminants rather than the amount of the target protein.

Speed is most important at the beginning of purification where contaminants such as proteases must be removed as quickly as possible.

Recovery becomes increasingly important as the purification proceeds because of the increased value of the purified product. Recovery is influenced by destructive processes in the sample and by unfavourable conditions on the column.

Resolution is achieved by the selectivity of the technique and the efficiency and selectivity of the chromatography matrix in producing narrow peaks. In general, resolution is most difficult to achieve in the final stages of purification when impurities and target protein are likely to have very similar properties.

Select a technique to meet the objectives for the purification step.

Choose logical combinations of purification techniques based on the main benefits of the technique and the condition of the sample at the beginning or end of each step.

A guide to the suitability of each purification technique for the stages in CIPP is shown in Table 4.2.

Table 4.2. Suitability of purification techniques for CIPP

	Typical characteristics		Purification phase				
Method	**Resolution**	**Capacity**	**Capture**	**Intermediate**	**Polishing**	**Sample start conditions**	**Sample end conditions**
AC	+++ or ++	+++ or ++	+++	++	+	Various binding conditions	Specific elution conditions
IMAC	+++	++	+++	++	+	Low concentration of imidazole, pH > 7.0	High concentration of imidazole, 500 mM NaCl, pH > 7.0
SEC	++	+	+		+++	Most conditions accepta-ble, limited sample volume	Buffer exchange possible, diluted sample
IEX	+++	+++	+++	+++	+++	Low ionic strength. pH depends on protein and IEX type	High ionic strength or pH changed
HIC	+++	++	++	+++	+++	High ionic strength, addition of salt required	Low ionic strength
RPC	+++	++		+	++	Ion-pair reagents and organic modifiers might be required	Organic solvents (risk for loss of biological activity)

Minimize sample handling between purification steps by combining techniques to avoid the need for sample conditioning. The product should be eluted from the first column in conditions suitable for the start conditions of the next column (see Table 4.2).

Ammonium sulfate, often used for sample clarification and concentration (see Appendix 1), leaves the sample in a high salt environment. Consequently HIC, which requires high salt to enhance binding to the media, becomes the excellent choice as the capture step. The salt concentration and the total sample volume will be significantly reduced after elution from the HIC column. Dilution of the fractionated sample or rapid buffer exchange using a desalting column will prepare it for the next IEX or AC step.

SEC is a nonbinding technique unaffected by buffer conditions, but with limited volume capacity. SEC is well-suited for use after any of the concentrating techniques (IEX, HIC, AC) since the target protein will be eluted in a reduced volume and the components from the buffer will not affect the size exclusion process.

Selection of the final strategy will always depend upon specific sample properties and the required level of purification. Logical combinations of techniques are shown in Figure 4.3.

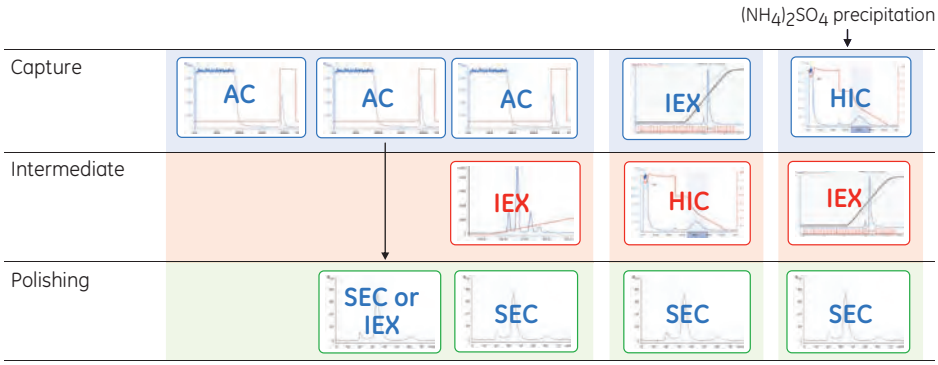


Fig 4.3. Examples of logical combinations of chromatography steps.

For any capture step, select the technique showing the most effective binding to the target protein while binding as few of the contaminants as possible, that is, the technique with the highest selectivity and/or capacity for the target protein.

A sample is purified using a combination of techniques and alternative selectivities. For example, in an IEX-HIC-SEC strategy, the capture step selects according to differences in charge (IEX), the intermediate purification step according to differences in hydrophobicity (HIC), and the final polishing step according to differences in size (SEC).

If nothing is known about the target protein, use IEX-HIC-SEC. This combination of techniques can be regarded as a standard protocol.

Consider the use of both anion and cation exchange chromatography to give different selectivities within the same purification strategy.

Ion exchange as a capture step

When IEX is used as a capture step, the objective is to quickly adsorb the protein(s) of interest from the crude sample and isolate them from critical contaminants such as proteases and glycosidases. The target protein(s) are concentrated and transferred to an environment which will conserve potency/activity. Removal of other critical contaminants may also be achieved by careful optimization of pH and elution conditions.

The focus is on capacity and speed in a capture step. It is advisable to compromise on the potential resolution that can be achieved by an IEX separation in order to maximize the capacity and/or speed of the separation in this first step.

IEX media for capture steps should offer high speed and high capacity. The choice of a suitable IEX medium depends on the sample properties and the scale of the purification (see also Chapter 5, Large-scale purification). Examples of IEX capture media include:

1. Sepharose Fast Flow (90 µm particle size) – good resolution for crude mixtures at any scale using flow velocities up to 300 cm/h and offering a wide range of selectivities.
2. Capto (90 µm particle size) - excellent pressure-flow properties for large-scale applications using flow velocities up to 700 cm/h.
3. Sepharose XL (90 µm particle size) – high capacity, good resolution for capture of selected proteins at laboratory and process scale using flow velocities up to 300 cm/h.
4. Sepharose Big Beads (200 µm particle size) – for viscous samples that preclude the use of IEX media with smaller particle size, using flows up to 300 cm/h, or for fast separations of very large sample volumes when resolution is of less importance, using flows up to 1000 cm/h.

If only milligram quantities of product are needed and the capture step will not be scaled up, use high-performance media such as SOURCE or Sepharose High Performance according to the capacity required.

Select start conditions that avoid adsorption of contaminants and so help to maximize the binding capacity for the target protein(s). This will facilitate a fast, simple step elution of the concentrated target protein(s).

Capture using Sepharose Q XL

Figure 4.4 shows optimization of a capture step used for purification of a recombinant enzyme, deacetooxycephalosporin C synthase (DAOCS). Since this enzyme is oxygen-sensitive, it was important to rapidly remove the most harmful contaminants from the relatively unstable target protein. The isoelectric point of DAOCS (pI = 4.8) made an anion exchanger the most suitable choice. Columns from the HiTrap IEX Selection Kit were screened to select the most suitable medium (results not shown) before optimizing the separation on a larger HiPrep Q XL 16/10 column. After a linear gradient elution (A), a multi-stepwise elution was tested (B). Since this caused a broader elution peak, it was decided to use a simple stepwise elution (C). In comparison with the gradient elution, the speed of the purification increased, buffer consumption decreased, and the target protein was eluted in a smaller volume (note that the x-axes differ between A and C).

Column:	HiPrep Q XL 16/10
Sample:	Clarified *E. coli* extract
Sample volume:	40 ml
Start buffer:	50 mM Tris-HCl, 1 mM EDTA, pH 7.5; 2 mM DTT, 200 mM benzamidine-HCl, 0.2 mM PMSF
Elution buffer:	Start buffer + 1 M NaCl
Flow rate (flow velocity):	10 ml/min (300 cm/h)
System:	ÄKTA system

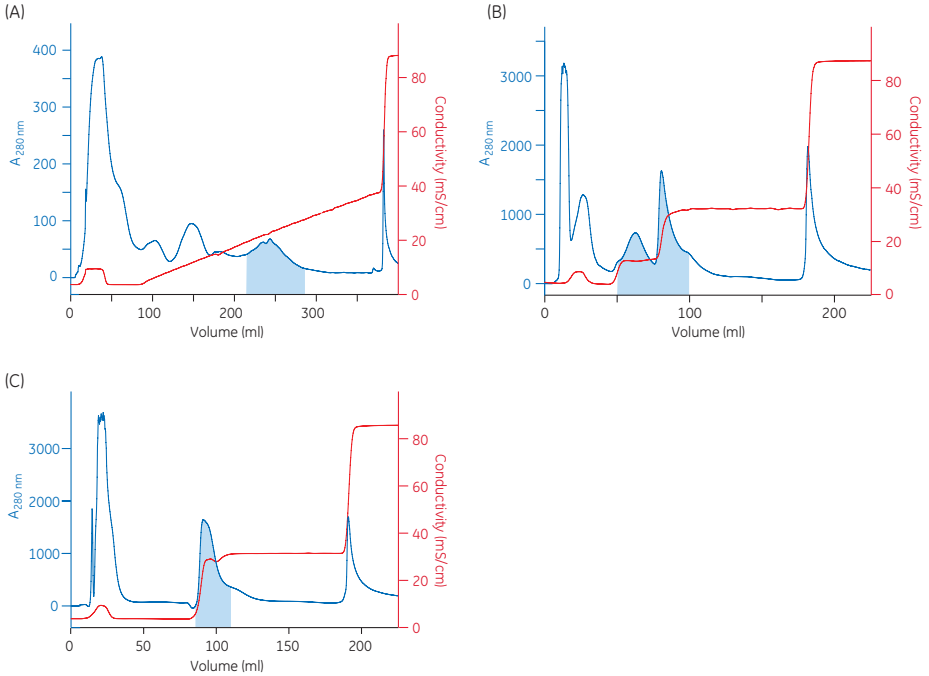


Fig 4.4. Capture step using IEX and optimization of conditions. The elution position of DAOCS is shaded.

Ion exchange for intermediate purification

When IEX is used for intermediate purification, the objective is to remove most of the significant impurities such as proteins, nucleic acids, endotoxins, and viruses.

In a typical intermediate purification step, speed is less critical since sample volume has been reduced and damaging contaminants have been removed during capture. Focus is on capacity and resolution in order to maintain productivity (amount of target protein processed per column in unit time) and to achieve as high selectivity (purity) as possible. Consequently, a gradient elution will usually be required.

Use a technique with a selectivity that is complementary to that used in the capture step.

IEX media for intermediate purification should offer high capacity and high resolution with a range of complementary selectivities The choice of a suitable IEX medium depends on the sample properties and the scale of the purification (see also Chapter 5, *Large-scale purification*). Examples of IEX media for intermediate purification:

1. Sepharose High Performance (34 µm particle size) – high resolution in laboratory scale using flow velocities up to 150 cm/h.
2. Capto ImpAct and Capto ImpRes (50 µm and 40 µm particle size) - excellent pressure-flow properties for large-scale applications using flow velocities up to 200 cm/h.
3. SOURCE 15 (15 µm particle size) – high throughput, high resolution for laboratory or large-scale applications using flow velocities up to 1800 cm/h.
4. SOURCE 30 (30 µm particle size) – an alternative to SOURCE 15 for large-scale applications when flow velocities up to 2000 cm/h can be used.
5. Sepharose Fast Flow (90 µm particle size) – fast separations using flow velocities up to 300 cm/h, broad range of selectivities.

If only milligram quantities are required and the intermediate purification step will not be scaled-up, use MonoBeads or MiniBeads according to the capacity required.

Optimize the selectivity of the medium to ensure high binding capacity and to maximize resolution. Use continuous gradient or multistep elution conditions.

Intermediate purification using Capto S ImpAct

Capto S ImpAct was used for intermediate purification in a three-step MAb-purification process (Fig 4.5).

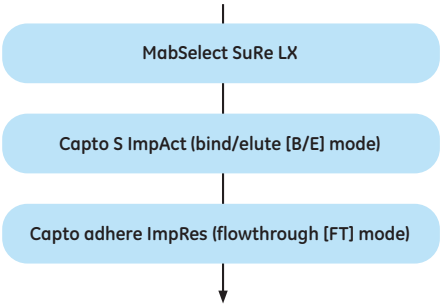


Fig 4.5. Three-step purification deploying MabSelect SuRe LX for capture, Capto S ImpAct for intermediate purification, and Capto Q for polishing.

The intermediate purification of MAb A resulted in reduction of aggregate concentration from 2% to 0.6% and host cell protein (HCP) concentration from 1800 ppm to 42 ppm. The yield of MAb A monomer in this step was 90%.

The high selectivity of Capto S ImpAct between MAb monomer, aggregates, and HCP can be seen from the chromatograms in Figure 4.6.

Column:	Tricorn 5/100
Medium:	Capto S ImpAct (B/E mode)
Sample:	MAb A in 50 mM sodium acetate, pH 5.3 (6.8 mS/cm)
Load:	64 g MAb/l medium (70% of Q_{B10})
Residence time:	5.4 min
Binding buffer:	50 mM sodium acetate, pH 5.3 (6.8 mS/cm)
Wash:	5 CV of binding buffer
Elution buffer:	50 mM sodium acetate, pH 5.3, 0 to 350 mM NaCl in 20 CV
System:	ÄKTA system

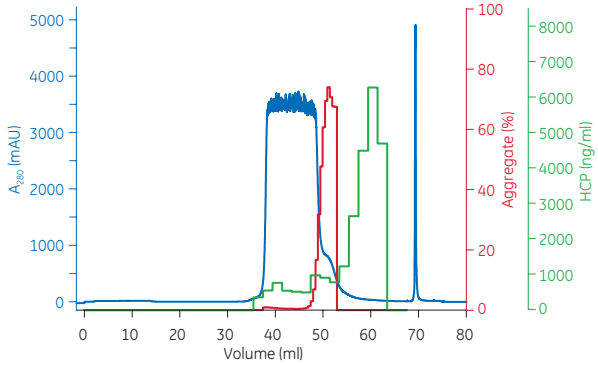


Fig 4.6. Intermediate purification of MAb A using Capto S ImpAct. Aggregates eluted in the tail of the elution peak (red histogram), whereas most of the HCP (green histogram) eluted after the elution peak (blue UV trace).

Ion exchange as a polishing step

When IEX is used for polishing, most impurities have been removed except for trace amounts or closely related substances such as structural variants of the target protein, nucleic acids, viruses, or endotoxins. The purpose of the separation is to reduce these variants and trace contaminants to acceptable levels for the application. In contrast to capture steps where a fast, high capacity, step elution is most commonly used, a polishing step will therefore focus on achieving the highest possible resolution. An example of this approach is shown in Figure 4.7 in which Mono S 5/50 GL was used to separate a recombinant DNA binding protein, transposase TniA, from minor contaminants remaining after partial purification by anion exchange and heparin affinity chromatography.

Column: Mono S 5/50 GL
Sample: 14.5 ml from HiPrep 26/10 Desalting
Start buffer: 20 mM MES pH 6.5, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT
Elution buffer: 20 mM MES pH 6.5, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 1 M NaCl
Flow rate: 1 ml/min
Gradient: 0% to 100% elution buffer, 20 CV

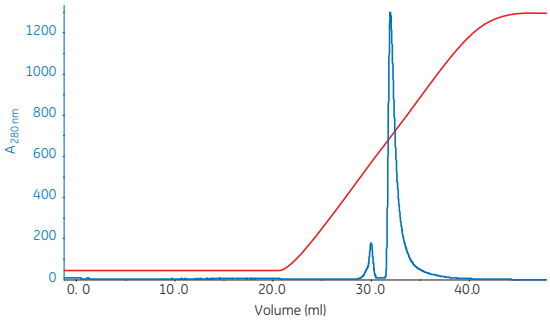


Fig 4.7. High-resolution cation exchange chromatography on Mono S 5/50 GL. The desalted sample was applied to Mono S 5/50 GL, and TniA was eluted as a sharp peak at approximately 500 mM NaCl.

IEX media for **polishing** steps should offer high resolution. The choice of a suitable IEX medium depends on the sample properties and the scale of the purification (see also Chapter 5, *Large-scale purification*). Examples of IEX polishing media:

1. MiniBeads (3 µm particle size) – polishing at microscale when highest resolution is essential.
2. MonoBeads (10 µm particle size) – polishing at laboratory scale when highest resolution is essential and a higher capacity than MiniBeads is required.
3. SOURCE 15 (15 µm particle size) – rapid, high resolution polishing for laboratory or large-scale applications using flow velocities up to 1800 cm/h.
4. SOURCE 30 (30 µm particle size) – an alternative to SOURCE 15 for large-scale applications when flow velocities up to 2000 cm/h can be used.
5. Sepharose High Performance (34 µm particle size) – high resolution at laboratory scale using flow velocities up to 150 cm/h.
6. Capto ImpAct and Capto ImpRes (50 µm and 40 µm particle size) - excellent pressure-flow properties for large-scale applications using flow velocities up to 200 cm/h.

Optimize the gradient elution to maximize selectivity. Use high efficiency media with small bead sizes to improve resolution.

Alternative techniques for polishing

Most commonly, separations by charge, hydrophobicity, or affinity will have been used in earlier stages of a purification strategy so that high-resolution SEC is ideal for the final polishing step. The product can be purified and transferred into the required buffer in one step and dimers and aggregates can be removed, as shown in Figure 4.6.

SEC is also the slowest of the chromatography techniques and the size of the column determines the volume of sample that can be applied. It is therefore most logical to use SEC after techniques that reduce sample volume so that smaller columns can be used. Media for polishing steps should offer the highest possible resolution. Superdex Increase is the first choice at laboratory scale and Superdex prep grade for large-scale applications.

Column:	XK 16/60 packed with Superdex 75 prep grade
Sample:	Partly purified ZZ-brain IGF
Sample load:	1.0 ml
Buffer:	300 mM ammonium acetate, pH 6.0
Flow rate (flow velocity):	0.5 ml/min (15 cm/h)

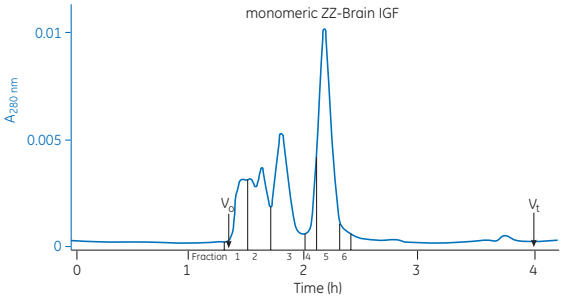


Fig 4.6. Final polishing step: separation of dimers and multimers on Superdex 75 prep grade.

RPC can also be considered for a polishing step, provided that the target protein can withstand the run conditions. RPC separates proteins and peptides on the basis of hydrophobicity and is a high selectivity (high resolution) technique, usually requiring the use of organic solvents. The technique is widely used for purity check analyses when recovery of activity and tertiary structure are not essential. Since many proteins are denatured by organic solvents, RPC is not generally recommended for protein purification because recovery of activity and return to a correct tertiary structure can be compromised. However, in the polishing phase, when the majority of protein impurities have been removed, RPC can be an excellent technique, particularly for small target proteins that are not often denatured by organic solvents.

Chapter 5
Large-scale purification

The BioProcess chromatography media family includes media widely used by biopharmaceutical manufacturers. Support for these products comprises of validated manufacturing methods, secure long-term chromatography media supply, safe and easy handling, and regulatory support files (RSF) to assist process validation and submissions to regulatory authorities. In addition, the Fast Trak Training and Education team provides high-level hands-on training for all key aspects of bioprocess development and manufacturing.

All BioProcess media have high chemical stability to allow efficient cleaning/sanitization procedures and validated packing methods established for a wide range of large-scale columns.

BioProcess media for IEX

BioProcess media for ion exchange chromatography are designed for large scale purification and use in industrial processes. Examples of BioProcess media for IEX purification are shown in Table 5.1 (the properties and handling of each medium are described in detail in Chapter 3).

Most of the media, except Sepharose Big Beads, are available in HiTrap and HiScreen formats for development of efficient and robust purification parameters before scaling up. Some of the media are also available in high-throughput process development (HTPD) formats, such as PreDictor 96-well filter plates for fast and easy parallel screening of running conditions and PreDictor RoboColumn (miniature columns for use with a robotic station) for testing of, for example, DBC. By using these small-scale formats in the early stages of process development, valuable time is saved and buffer and sample consumption reduced.

The range of BioProcess media includes capture media such as Capto, Sepharose Fast Flow, and Sepharose XL. These media can be used at high flow rates and have high dynamic binding capacities. Media with smaller beads, such as Capto ImpRes, Capto ImpAct, Sepharose High Performance, and SOURCE give high final resolution and are suitable for polishing purification (see also Chapter 4 for the principle of capture and polishing purification).

Table 5.1. Examples of BioProcess chromatography media for IEX

Medium	Chromatography method	Average particle size, d_{50v} (µm)	Recommended flow rate[1]	Protein binding capacity[1]	Main usage
Q Sepharose Big Beads	Strong anion	200	High	low	Capture
SP Sepharose Big Beads	Strong cation	200	High	low	Capture
Capto Q	Strong anion	90	High	High	Capture
Capto DEAE	Weak anion	90	High	Medium	Capture
Capto S	Strong cation	90	High	High	Capture
Q Sepharose Fast Flow	Strong anion	90	Medium	Medium	Capture
DEAE Sepharose Fast Flow	Weak anion	90	Medium	Medium	Capture
ANX Sepharose 4 Fast Flow (high sub)	Weak anion	90	Medium	Medium	Capture
SP Sepharose Fast Flow	Strong cation	90	Medium	Medium	Capture
CM Sepharose Fast Flow	Weak cation	90	Medium	Medium	Capture
Q Sepharose XL	Strong anion	90	Medium	High	Capture
SP Sepharose XL	Strong cation	90	Medium	High	Capture
Capto Q ImpRes	Strong anion	40	Medium	Medium	Polishing
Capto SP ImpRes	Strong cation	40	Medium	Medium	Polishing
Capto S ImpAct	Strong cation	50	Medium	High	Polishing
SOURCE 30Q	Stong anion	30	High	Medium	Polishing
SOURCE 30S	Strong cation	30	High	Medium	Polishing
SOURCE 15Q	Strong anion	15	High	Medium	Polishing
SOURCE 15S	Strong cation	15	High	Medium	Polishing

[1] *Recommended flow rate and protein binding capacity are expressed as high, medium, or low for easy comparison. The values for each medium can be found in Chapter 3.*

Capture purification

General capture purification is performed using Capto and Sepharose Fast Flow. When IEX is used as a capture step, the objective is to quickly adsorb the protein of interest from the crude sample and remove critical contaminants such as proteases. For modern industrial applications, Capto media are usually the preferred option having benefits such as high rigidity and ability to run at high flow rates. Figure 5.1 shows a comparison of media properties. Capto media give increased DBC over a wide range of residence times due to excellent mass transfer properties. Further, a high DBC contributes to shortening the overall processing time as the total number of cycles can be reduced.

Sepharose Fast Flow media are available with a wide range of ion exchange groups and are still widely used in biopharmaceutical processes.

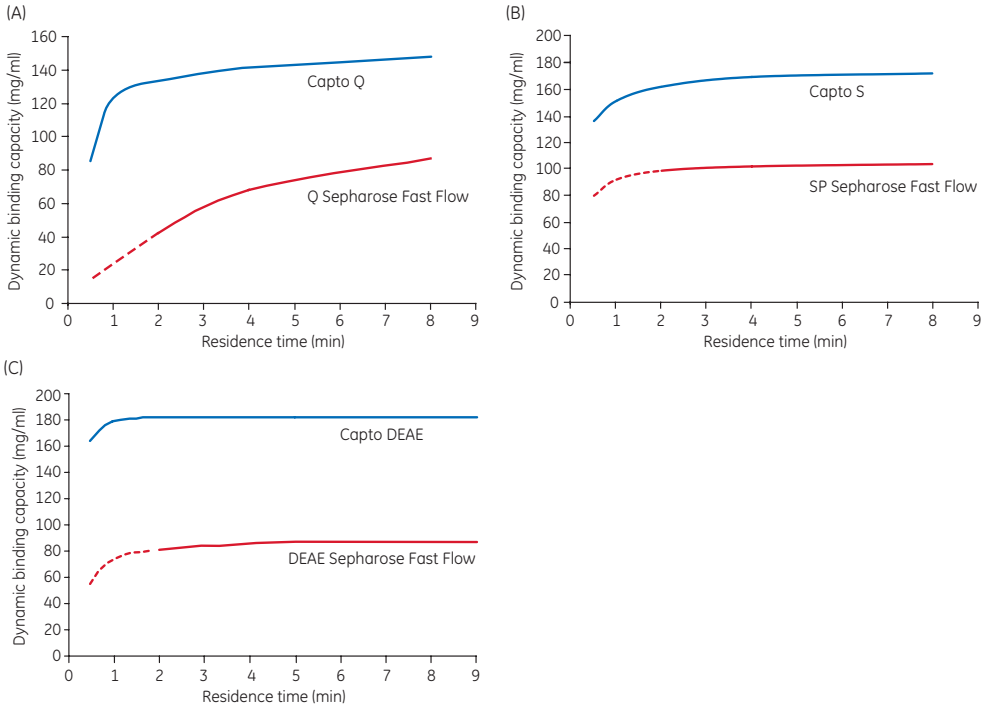


Fig 5.1. Dynamic binding capacity (DBC) as a function of residence time for Capto and corresponding Sepharose Fast Flow media. Proteins: (A) BSA; (B) α-chymotrypsin; (C) amyloglucosidase. Residence times below 2 min are not possible for Sepharose Fast Flow in large-scale columns due to lower pressure/flow properties than for Capto media.

The capture media Q Sepharose XL and SP Sepharose XL have greater binding capacity compared with other Sepharose media. The high binding capacity has been obtained by binding the ionic group to long, flexible dextran chains coupled to the agarose matrix.

Sepharose Big Beads are designed for capture of large volumes of crude and/or viscous sample. Due to the large bead size of the Sepharose Big Beads matrix (200 µm average particle size), a lower resolution compared with other capture media can be expected.

Polishing purification

The polishing purification is performed using chromatography media with smaller average bead size, such as Capto ImpRes, Capto ImpAct, and SOURCE media. In contrast to capture purification where a fast, high-capacity step elution is most commonly used, a polishing purification will focus on achieving the high resolution (resulting in high purity).

Although the charged groups of the S, SP, Q, and DEAE ligands are identical between different IEX media, minor differences in selectivity can occur due to differences in base matrix, ligand density, ligand composition, and surface extenders. Capto ImpRes media are designed for high resolution and high throughput during polishing steps. A comparison with Q Sepharose High Performance in HiScreen columns showed similar resolution results, although HiScreen Capto Q ImpRes was run at 300 cm/h—twice the flow velocity of HiScreen Q HP (Fig 5.2). Capto ImpRes media provide improved performance over Sepharose when scaling up due to their good pressure/flow properties, allowing high flow rates.

Columns:	HiScreen Capto Q ImpRes, HiScreen Q HP, 4.7 ml
Sample:	5 ml apo-transferrin (0.3 mg/mL), α-lactoalbumin (0.4 mg/ml), soybean trypsin inhibitor (0.6 mg/ml) in start buffer
Start buffer:	50 mM Tris, pH 7.4
Elution buffer:	50 mM Tris, 500 mM NaCl, pH 7.4
Flow rate (flow velocity):	HiScreen Capto Q ImpRes, 2.3 ml/min (300 cm/h); HiScreen Q HP, 1.2 ml/min (150 cm/h)
Gradient:	0% to 100% elution buffer in 20 CV
Residence time:	HiScreen Capto Q ImpRes, 2 min; HiScreen Q HP, 4 min

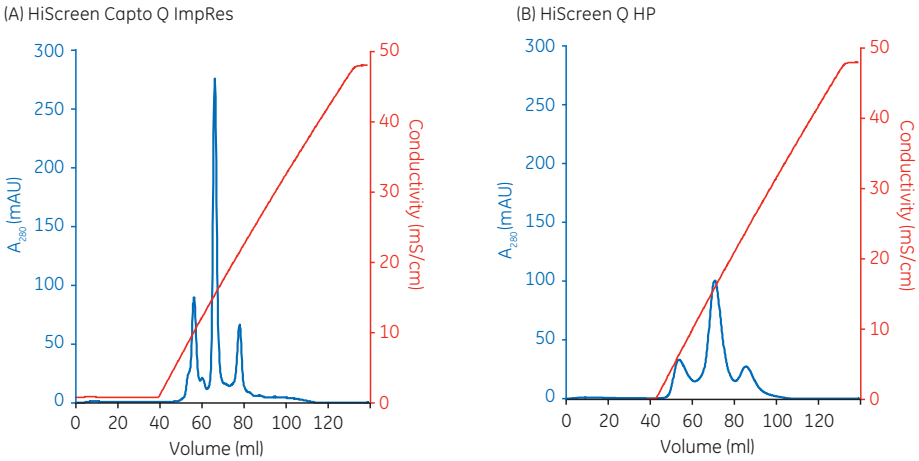


Fig 5.2. Chromatograms from resolution comparisons on two IEX media with the same Q charged group ligand. Peaks (left to right) are apo-transferrin, α-lactoalbumin, and soybean trypsin inhibitor. The small bead size of (A) Capto Q ImpRes and (B) Q Sepharose High Performance gives high resolution in both cases. However, the higher flow velocity possible with Capto Q ImpRes makes it a good choice for large-scale intermediate purification or polishing.

The difference between Capto ImpRes and Sepharose High Performance media is also illustrated in Figure 5.3. Although the bead sizes of the media are similar, the pressure/flow properties of Capto ImpRes are significantly improved as a result of the greater mechanical stability of its high-flow base matrix.

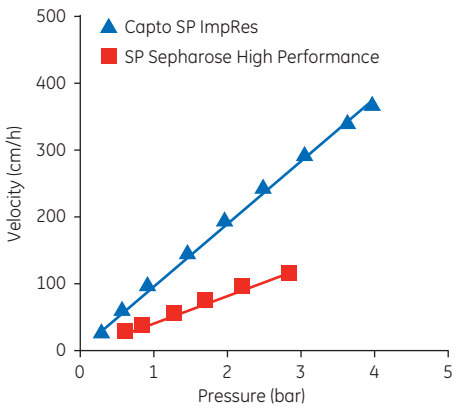


Fig 5.3. The pressure-flow properties of Capto ImpRes are enhanced compared with Sepharose High Performance due to the improved mechanical stability of the base matrix. Running conditions: AxiChrom 300 column, 20 cm bed height with water at 20°C.

Capto S ImpAct chromatography medium is designed for the polishing steps of MAb and a wide range of other biomolecules. In this study, the binding capacity for MAb A and the efficiency in the clearance of impurities was evaluated. The content of aggregates in the sample was approximately 7% after the initial protein A capture step. A high load, 80 mg/ml medium, was applied to the Capto S ImpAct column and elution was performed using a linear gradient. The chromatogram illustrates that aggregates (green) elute at the tail of the elution peak (Fig 5.4). The purification resulted in 90% monomer recovery and reduction of aggregate content to 0.9%.

Column:	HiScreen Capto S ImpAct, CV 4.7 ml
Sample:	~ 10 mg/mL MAb A purified on MabSelect SuRe, buffer exchanged into start buffer
Sample load:	80 mg/ml medium
Start buffer:	50 mM sodium acetate, pH 5.0 + 50 mM NaCl
Elution buffer:	50 mM sodium acetate, pH 5.0 + 500 mM NaCl
Flow rate loading:	1.1 ml/min, residence time 4 min
Flow rate elution:	0.6 ml/min, residence time 8 min
Gradient:	Linear, 20 CV

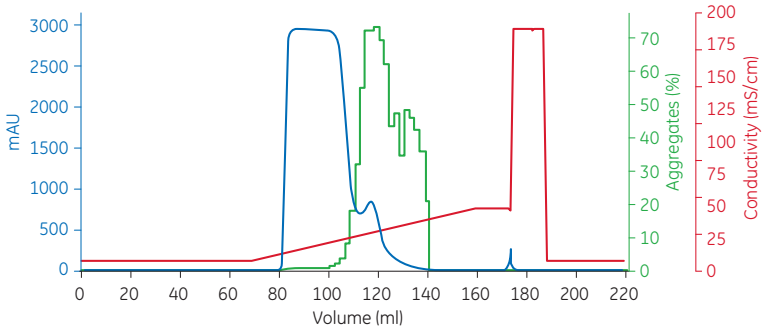


Fig 5.4. Purification of MAb A at a high sample load (80 mg/ml medium). Histogram in green represents aggregates in fractions.

SOURCE 30Q and 30S are easy to pack at both laboratory and large scale and are suitable for industrial processes when large volumes of partially purified material need to be processed. The excellent scalability of SOURCE 30S was shown in a scale up from a 105-ml small scale-column to a 50-l custom-designed production column (Fig 5.5). The results show that performance was maintained despite high flow rates and an almost 500-fold scale-up factor.

Column:	SOURCE 30S: (A) FineLINE Pilot 35, 35 mm i.d. × 109 mm (105 ml)
	SOURCE 30S: (B) FineLINE 100, 100 mm i.d. × 100 mm (0.78 l)
	SOURCE 30S: (C) FineLINE 800, 800 mm i.d. × 100 mm (50 l)
Sample:	Ribonuclease A, cytochrome C, and lysozyme (3.75:1:1)
Sample load:	0.32 mg total protein/ml media
Start buffer:	20 mM sodium phosphate, pH 6.8
Elution buffer:	20 mM sodium phosphate + 400 mM NaCl, pH 6.8
Flow rate:	(A) 48 ml/min (B) 0.39 l/min, (C) 25 l/min
Gradient:	0% to 100% elution buffer, 20 CV

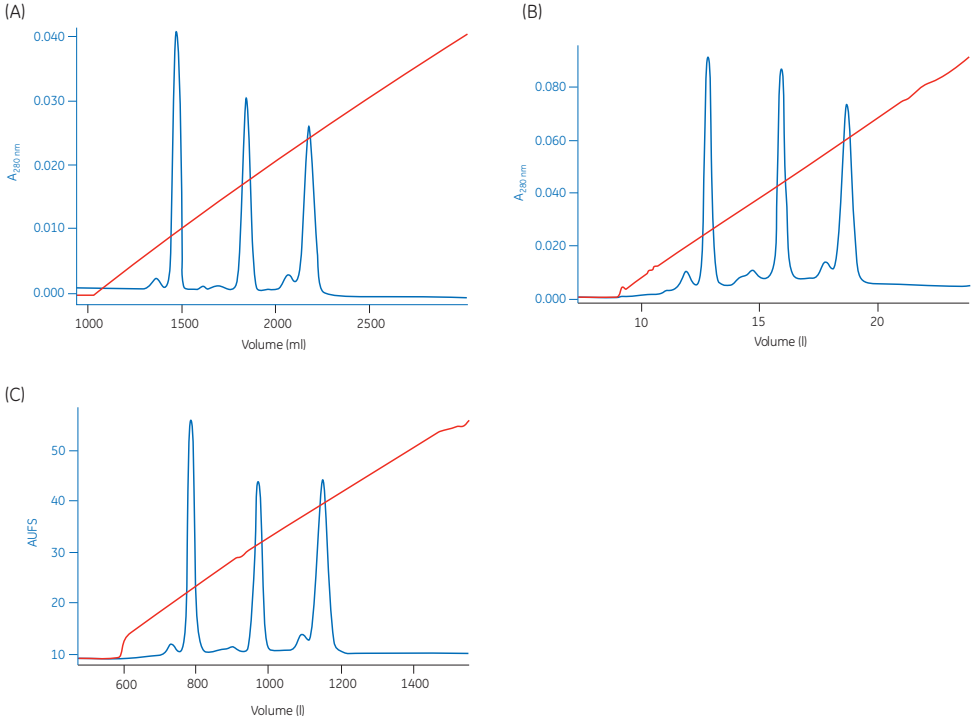


Fig 5.5. Scale-up from a FineLINE Pilot 35 column via FineLINE 100 column (7-fold) to FineLine 800 custom-designed column (64-fold). Total scale-up factor: 476-fold.

SOURCE 15Q and SOURCE 15S have a uniform, 15 µm diameter, spherical shape and are designed for high-resolution purification at lab scale and for scaling up. The beads give stable packed beds with low back pressure. Figure 5.6 illustrates the maintained performance of high-resolution separations also at very high flow rates.

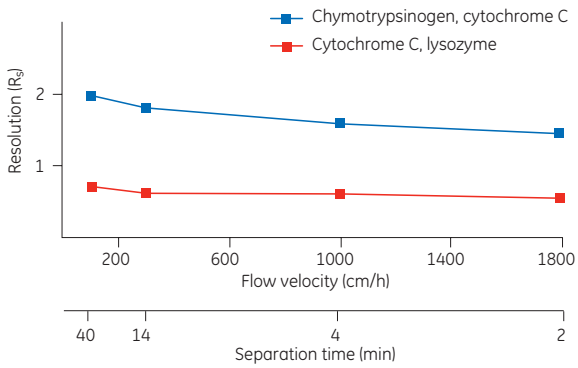


Fig 5.6. Resolution vs flow velocity for model proteins. Column: RESOURCE S, 1 ml (6.4 mm diameter × 30 mm bed height). Sample: chymotrypsinogen, cytochrome C, lysozyme. Total load 16 mg.

Prepacked, disposable solutions speed up the downstream process

In addition to a wide range of industrial-scale columns such as AxiChrom columns and bulk media for purification of proteins, GE offers large-scale, disposable ReadyToProcess columns. These columns are prepacked, prequalified, and presanitized process chromatography columns available with a range of BioProcess media—including Capto, Capto ImpAct, Capto ImpRes, Sepharose Fast Flow, and Sepharose High Performance product families. ReadyToProcess columns are available in several different sizes (Fig 5.7) and are designed for purification of biopharmaceuticals (e.g., proteins and antibodies, vaccines, plasmids, and viruses) for clinical phase I and II studies. Depending on the scale of operations, the columns can also be used for manufacturing, as well as preclinical studies. ReadyToProcess columns make column packing, qualification, and sanitization redundant in the purification process allowing significant time savings.

Fig 5.7. ReadyToProcess columns are easily connected to the system and can be disposed after completed production.

Custom Designed Media

Custom Designed Media (CDM) can be produced for specific industrial process separations when suitable media are not available from the standard range. The Custom Designed Media group (CDM group) works in close collaboration with the user to design, manufacture, test, and deliver media for specialized purification requirements.

See also H*andbook of Process Chromatography: Optimization, Scale-up, and Validation*, 18112156.

Appendix 1
Sample preparation

Samples for chromatographic purification should be clear and free from particulate matter. Simple steps to clarify a sample before beginning purification will avoid clogging the column, reduce the need for stringent washing procedures, and extend the life of the chromatographic medium.

Sample extraction procedures and the selection of buffers, additives, and detergents are determined largely by the source of the material, the stability of the target molecule, the chromatographic techniques that will be employed and the intended use of the product. These subjects are dealt with in general terms in the *Strategies for Protein Purification Handbook* and more specifically according to target molecule in the *Recombinant Protein Purification Handbook,* and *Antibody Purification Handbook*, available from GE.

Sample stability

In the majority of cases, biological activity needs to be retained after purification. Retaining the activity of the target molecule is also an advantage when following the progress of the purification, since detection of the target molecule often relies on its biological activity. Denaturation of sample components often leads to precipitation or enhanced nonspecific adsorption, both of which will impair column function. Hence there are many advantages to checking the stability limits of the sample and working within these limits during purification.

Proteins generally contain a high degree of tertiary structure, kept together by van der Waals' forces, ionic and hydrophobic interactions, and hydrogen bonding. Any conditions capable of destabilizing these forces may cause denaturation and/or precipitation. By contrast, peptides contain a low degree of tertiary structure. Their native state is dominated by secondary structures, stabilized mainly by hydrogen bonding. For this reason, peptides tolerate a much wider range of conditions than proteins. This basic difference in native structures is also reflected in that proteins are not easily renatured, while peptides often renature spontaneously.

It is advisable to perform some stability tests before beginning to develop a purification protocol. The list below shows examples of such testing:

- Test pH stability in steps of one pH unit between pH 2.0 and pH 9.0.
- Test salt stability with 0 to 2 M NaCl and 0 to 2 M $(NH_4)_2SO_4$ in steps of 0.5 M.
- Test the stability towards acetonitrile and methanol in 10% steps between 0% and 50%.
- Test the temperature stability in 10°C steps from 4°C to 40°C.
- Test the stability and occurrence of proteolytic activity by leaving an aliquot of the sample at room temperature overnight. Centrifuge each sample and measure activity and UV absorbance at 280 nm in the supernatant.

Sample clarification

Centrifugation and filtration are standard laboratory techniques for sample clarification and are used routinely when handling small samples.

It is highly recommended to centrifuge and filter samples immediately before chromatographic purification.

Centrifugation

Centrifugation removes lipids and particulate matter, such as cell debris. If the sample is still not clear after centrifugation, use filter paper or a 5 µm filter as a first step and one of the filters below as a second-step filter.

- For small sample volumes or proteins that adsorb to filters, centrifuge at 10 000 × g for 15 min.
- For cell lysates, centrifuge at 40 000 to 50 000 × g for 30 min.
- Serum samples can be filtered through glass wool after centrifugation to remove remaining lipids.

Filtration

Filtration removes particulate matter. Whatman™ syringe filters, which give the least amount of nonspecific binding of proteins, are composed of cellulose acetate (CA), regenerated cellulose (RA), or polyvinylidene fluoride (PVDF) (Table A1.1).

Table A1.1. Whatman syringe filters for filtration of samples

Filter pore size (µm)	Up to sample volume (ml)	Whatman syringe filter[1]	Membrane
0.8	100	Puradisc FP 30	CA
0.45	1	Puradisc 4	PVDF
0.45	10	Puradisc 13	PVDF
0.45	100	Puradisc 25	PVDF
0.45	10	SPARTAN™ 13	RC
0.45	100	SPARTAN 30	RC
0.45	100	Puradisc FP 30	CA
0.2	1	Puradisc 4	PVDF
0.2	10	Puradisc 13	PVDF
0.2	100	Puradisc 25	PVDF
0.2	10	SPARTAN 13	RC
0.2	100	SPARTAN 30	RC
0.2	100	Puradisc FP 30	CA

[1] *The number indicates the diameter (mm) of the syringe filter.*

For sample preparation before chromatography, select a filter pore size in relation to the bead size of the chromatographic medium (Table A1.2).

Table A1.2. Selecting a sample filter based on the bead size of the chomatographic medium used

Nominal pore size of filter (µm)	Particle size of chromatographic medium (µm)
1.0	90 and upwards
0.45	30 or 34
0.22	3, 10, 15 or when extra clean samples or sterile filtration is required

Check the recovery of the target protein in a test run. Some proteins adsorb nonspecifically to filter surfaces.

Desalting

Desalting columns are suitable for any sample volume and will rapidly remove low molecular weight contaminants in a single step at the same time as transferring the sample into the correct buffer conditions. Centrifugation and/or filtration of the sample before desalting is still recommended. Detailed procedures for buffer exchange and desalting are given in the section *Buffer exchange and desalting* later in this appendix.

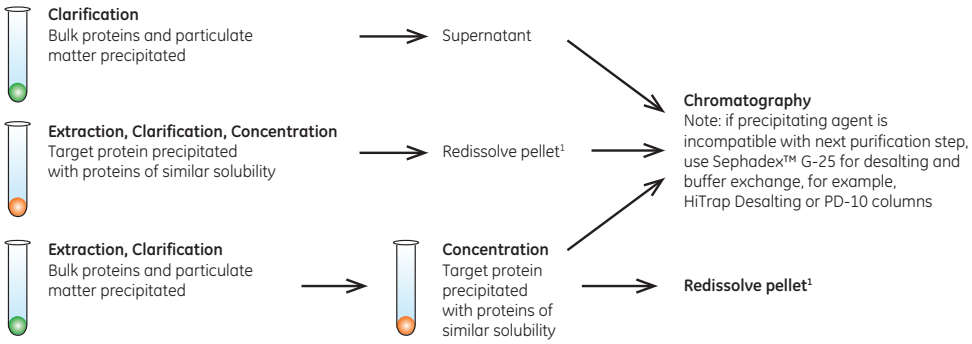
At laboratory scale, when samples are reasonably clean after filtration or centrifugation, the buffer exchange and desalting step can be avoided. For affinity chromatography or hydrophobic interaction chromatography, it might be sufficient to adjust the pH of the sample. For IEX, it might be sufficient to dilute the sample to reduce the ionic strength.

Specific sample preparation steps

Specific sample preparation steps might be required if the crude sample is known to contain contamininants such as lipids, lipoproteins, or phenol red that might build up on a column. Gross impurities, such as bulk protein, should be removed before any chromatographic step.

Fractional precipitation

Fractional precipitation is occasionally used at laboratory scale to remove gross impurities from the sample. Precipitation techniques separate fractions by the principle of differential solubility. Because proteins differ in their degree of hydrophobicity, increased salt concentrations can enhance hydrophobic interactions between the proteins and cause precipitation. Fractional precipitation can be applied to remove gross impurities in three different ways, as shown in Figure A1.1.



[1] *Remember: not all proteins are easy to redissolve, yield can be reduced*

Fig A1.1. Three ways to use precipitation.

Precipitation techniques can be affected by temperature, pH, and sample concentration. These parameters should be controlled to ensure reproducible results.

Examples of precipitation agents are reviewed in Table A1.3. The most common precipitation method using ammonium sulfate is described in more detail.

Table A1.3. Examples of precipitation techniques

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulfate	As described below.	> 1 mg/ml proteins especially immuno-globulins.	Stabilizes proteins, no denaturation, supernatant can go directly to HIC. Helps to reduce lipid content.
Dextran sulfate	Add 0.04 ml 10% dextran sulfate and 1 ml of 1 M $CaCl_2$ per ml sample, mix 15 min, centrifuge 10 000 × g, discard pellet.	Samples with high levels of lipoprotein, e.g., ascites.	Precipitates lipoprotein.
Polyvinylpyrrolidine	Add 3% (w/v), stir 4 h, centrifuge 17 000 × g, discard pellet.	Samples with high levels of lipoprotein, e.g., ascites.	Alternative to dextran sulfate.
Polyethylene glycol (PEG, M_r > 4000)	Up to 20% w/v.	Plasma proteins.	No denaturation, supernatant goes directly to IEX or AC, complete removal might be difficult. Stabilizes proteins.
Acetone (cold)	Up to 80% v/v at ± 0°C. Collect pellet after centrifugation at full speed in a microcentrifuge.		Can denature protein irreversibly. Useful for peptide precipitation or concentration of sample for electrophoresis.
Polyethyleneimine	0.1% w/v.		Precipitates aggregated nucleoproteins.
Protamine sulfate	1% w/v.		Precipitates aggregated nucleoproteins.
Streptomycin sulfate	1% w/v.		Precipitation of nucleic acids.
Caprylic acid	(X/15) g where X = volume of sample.	Antibody concentration should be > 1 mg/ml.	Precipitates bulk of proteins from sera or ascites, leaving immunoglobulins in solution.

Details taken from: Scopes R. K., Protein Purification, Principles and Practice, Springer, (1994), J. C. Janson and L. Rydén, Protein Purification, Principles, High Resolution Methods and Applications, second ed. Wiley Inc, (1998). Personal communications.

Ammonium sulfate precipitation

Some proteins can be damaged by ammonium sulfate. Take care when adding crystalline ammonium sulfate; high local concentrations can cause contamination of the precipitate with unwanted proteins.

For routine, reproducible purification, precipitation with ammonium sulfate should be avoided in favor of chromatography.

In general, precipitation is rarely effective for protein concentrations below 1 mg/ml.

Solutions needed for precipitation:

Saturated ammonium sulfate solution (add 100 g ammonium sulfate to 100 ml distilled water, stir to dissolve).

1 M Tris-HCl, pH 8.0.

Buffer for first purification step.

1. Filter (0.45 µm) or centrifuge the sample (10 000 × g at 4°C).
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add ammonium sulfate solution, drop by drop. Add up to 50% saturation[1]. Stir for 1 h.
4. Centrifuge 20 min at 10 000 × g.
5. Remove supernatant. Wash the pellet twice by resuspension in an equal volume of ammonium sulfate solution of the same concentration (i.e. a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again.
6. Dissolve pellet in a small volume of the buffer to be used for the next step.
7. Ammonium sulfate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns (see *Buffer exchange and desalting* later in this appendix).

[1] *The percent saturation can be adjusted either to precipitate a target molecule or to precipitate contaminants.*

The quantity of ammonium sulfate required to reach a given degree of saturation varies according to temperature. Table A1.4 shows the quantities required at 20°C.

Table A1.4. Quantities of ammonium sulfate required to reach given degrees of saturation at 20°C

	Final percent saturation to be obtained																
	20	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**85**	**90**	**95**	**100**
Starting percent saturation	**Amount of ammonium sulfate to add (gram) per liter of solution at 20°C**																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Resolubilization of protein precipitates

Many proteins are easily resolubilized in a small amount of the buffer to be used in the next chromatographic step. However, a denaturing agent may be required for less soluble proteins. Specific conditions will depend upon the specific protein. These agents must always be removed to allow complete refolding of the protein and to maximize recovery of mass and activity. A chromatographic step often removes a denaturant during purification. Table A1.5 gives examples of common denaturing agents.

Table A1.5. Denaturing agents used for resolubilization of relatively insoluble proteins

Denaturing agent	Typical conditions for use (molar, M)	Removal/comment
Urea	2 to 8	Remove using Sephadex G-25
Guanidine hydrochloride	3 to 6	Remove using Sephadex G-25

Details taken from: Scopes R. K., Protein Purification, Principles and Practice, Springer, (1994), J. C. Janson and L. Rydén, Protein Purification, Principles, High Resolution Methods and Applications, second ed. Wiley Inc, (1998) and other sources.

Buffer exchange and desalting

Dialysis is frequently mentioned in the literature as a technique to remove salt or other small molecules and to exchange the buffer composition of a sample. However, dialysis is generally a very slow technique, requiring large volumes of buffer. There is also a risk of losing material during handling or as a result of proteolytic breakdown or nonspecific binding to the dialysis membranes. A simpler and much faster technique is to use a desalting column, packed with Sephadex G-25, to perform a group separation between high and low molecular weight substances. Proteins are separated from salts and other small molecules.

In a fast, single step, the sample is desalted, transferred into a new buffer and low molecular weight materials are removed.

Desalting columns are used not only to remove low molecular weight contaminants, such as salt, but also for buffer exchange before or after different chromatographic steps and for the rapid removal of reagents to terminate a reaction.

Sample volumes up to 30% of the total volume of the desalting column can be processed. Sample concentration does not influence the separation as long as the concentration of proteins does not exceed 70 mg/ml when using normal aqueous buffers. The sample should be fully dissolved. Centrifuge or filter to remove particulate material.

For small sample volumes, it is possible to dilute the sample with the start buffer that is to be used for chromatographic purification, but cell debris and particulate matter must still be removed.

Volatile buffers such as 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate can be used if it is necessary to avoid the presence of NaCl.

Figure A1.2 shows a typical buffer exchange and desalting separation. The process can be monitored by following changes in UV absorption and conductivity.

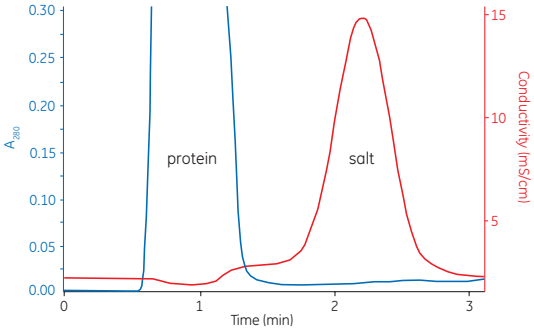


Fig A1.2. Buffer exchange of mouse plasma (10 ml) on HiPrep 26/10 Desalting.

For laboratory-scale operations, Table A1.6 shows examples of prepacked, ready-to-use desalting and buffer exchange columns (see *Size Exclusion Chromatography Handbook*, 18102218, for additional formats).

Table A1.6. Examples of desalting and buffer exchange columns

Column	Sample volume (ml)	Sample elution volume (ml)
PD MiniTrap™ G-25	0.2 to 0.5	0.1 to 0.5
PD-10 (gravity feed column)	1.5 to 2.5	2.5 to 3.5
HiTrap Desalting, 5 ml	0.25 to 1.5	1.0 to 2.0
HiPrep 26/10 Desalting	2.5 to 15	7.5 to 20

To desalt larger sample volumes:

- Connect up to five HiTrap Desalting 5 ml columns in series to increase the sample volume capacity, for example, two columns: sample volume 3 ml, five columns: sample volume 7.5 ml.
- Connect up to four HiPrep 26/10 Desalting columns in series to increase the sample volume capacity, for example two columns: sample volume 30 ml, four columns: sample volume 60 ml. Even with four columns in series, the sample can be processed in 20 to 30 min, at room temperature, in aqueous buffers.

Instructions are supplied with each column. Desalting and buffer exchange can take less than 5 min per sample with greater than 95% recovery for most proteins.

Manual desalting with HiTrap Desalting 5 ml using a syringe

1. Fill the syringe with buffer. Remove the stop plug. To avoid introducing air into the column, connect the column "drop to drop" to the syringe (via the adapter provided).
2. Remove the snap-off end.
3. Wash the column with 25 ml buffer at 5 ml/min to remove completely the 20% ethanol (supplied as storage buffer). If air is trapped in the column, wash with degassed buffer until the air disappears. Air bubbles introduced onto the column by accident during sample application do not influence the separation.
4. Apply the sample (0.25 to 1.5 ml) using a 2 to 5 ml syringe at a flow rate between 1 to 10 ml/min. Discard the liquid eluted from the column.
5. If the sample volume is less than 1.5 ml, change to buffer and proceed with the injection until a total of 1.5 ml has been eluted. Discard the eluted liquid.
6. Elute the protein with the appropriate volume selected from Table A1.7 and collect the desalted protein.

Note: 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column. A simple peristaltic pump or a chromatography system can also be used for the desalting procedure.

The maximum recommended sample volume is 1.5 ml. See Table A1.7 for the effect of reducing the sample volume applied to the column.

Table A1.7. Recommended sample and elution volumes using a syringe

Sample load (ml)	Add buffer (ml)	Elute and collect (ml)	Yield (%)	Remaining salt (%)	Dilution factor
0.25	1.25	1.0	> 95	0.0	4.0
0.50	1.0	1.5	> 95	< 0.1	3.0
1.00	0.5	2.0	> 95	< 0.2	2.0
1.50	0	2.0	> 95	< 0.2	1.3

Removal of lipoproteins

Lipoproteins and other lipid material can rapidly clog chromatography columns and it is advisable to remove them before beginning purification. Precipitation agents such as dextran sulfate and polyvinylpyrrolidine, described under Fractional precipitation above, are recommended to remove high levels of lipoproteins from samples such as ascitic fluid.

Centrifuge samples when performing precipitation to avoid the risk of nonspecific binding of the target molecule to a filter.

Samples such as serum can be filtered through glass wool to remove remaining lipids.

Removal of phenol red

Phenol red is frequently used at laboratory scale as a pH indicator in cell culture. Although not directly interfering with purification, phenol red binds to certain purification media and should be removed as early as possible to avoid the risk of contamination. It is known to bind to anion exchange media at pH > 7.0.

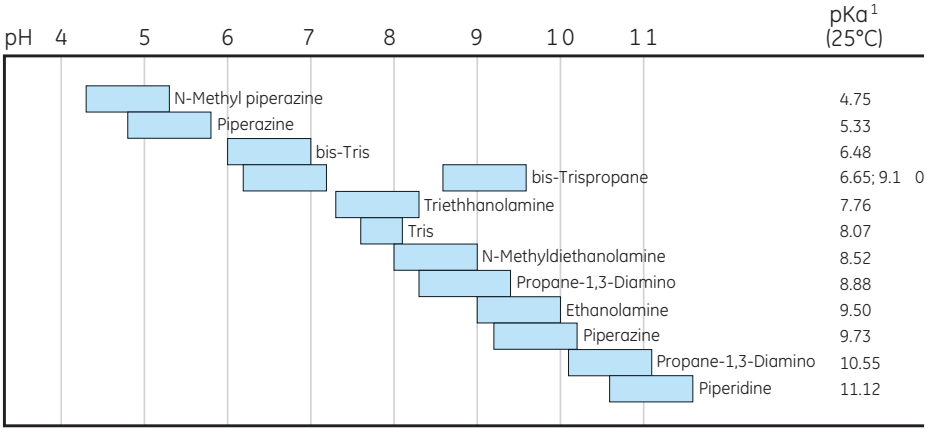
Use a desalting column to simultaneously remove phenol red (a low molecular weight molecule) and transfer sample to the correct buffer conditions for further purification, as described under *Buffer exchange and desalting* earlier in this appendix.

Removal of low molecular weight contaminants

If samples contain a high level of low molecular weight contaminants, use a desalting column before the first chromatographic purification step, as described under *Buffer exchange and desalting* earlier in this appendix.

Appendix 2
Nonvolatile and volatile buffer systems

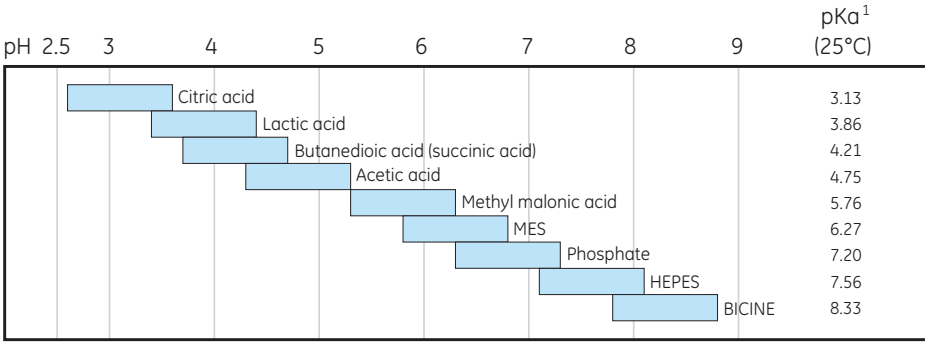
Nonvolatile buffers for anion exchange chromatography



pH interval	Substance	Conc. (mM)	Counterion	pKa (25°C)[1]	d(pKa)/dT (°C)
4.3 to 5.3	N-Methylpiperazine	20	Cl^-	4.75	-0.015
4.8 to 5.8	Piperazine	20	Cl^- or $HCOO^-$	5.33	-0.015
5.5 to 6.5	L-Histidine	20	Cl^-	6.04	
6.0 to 7.0	bis-Tris	20	Cl^-	6.48	-0.017
6.2 to 7.2; 8.6 to 9.6	bis-Tris propane	20	Cl^-	6.65; 9.10	
7.3 to 8.3	Triethanolamine	20	Cl^- or CH_3COO^-	7.76	-0.020
7.6 to 8.6	Tris	20	Cl^-	8.07	-0.028
8.0 to 9.0	N-Methyldiethanolamine	20	SO_4^{2-}	8.52	-0.028
8.0 to 9.0	N-Methyldiethanolamine	50	Cl^- or CH_3COO^-	8.52	-0.028
8.4 to 9.4	Diethanolamine	20 at pH 8.4 50 at pH 8.8	Cl^-	8.88	-0.025
8.4 to 9.4	Propane 1,3-Diamino	20	Cl^-	8.88	-0.031
9.0 to 10.0	Ethanolamine	20	Cl^-	9.50	-0.029
9.2 to 10.2	Piperazine	20	Cl^-	9.73	-0.026
10.0 to 11.0	Propane 1,3-Diamino	20	Cl^-	10.55	-0.026
10.6 to 11.6	Piperidine	20	Cl^-	11.12	-0.031

[1] *Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002 to 2003.*

Nonvolatile buffers for cation exchange chromatography



pH interval	Substance	Conc. (mM)	Counterion	pKa (25°C)[1]	d(pKa)/dT (°C)
1.4 to 2.4	Maleic acid	20	Na^+	1.92	
2.6 to 3.6	Methyl malonic acid	20	Na^+ or Li^+	3.07	
2.6 to 3.6	Citric acid	20	Na^+	3.13	-0.0024
3.3 to 4.3	Lactic acid	50	Na^+	3.86	
3.3 to 4.3	Formic acid	50	Na^+ or Li^+	3.75	+0.0002
3.7 to 4.7; 5.1 to 6.1	Succinic acid	50	Na^+	4.21; 5.64	-0.0018
4.3 to 5.3	Acetic acid	50	Na^+ or Li^+	4.75	+0.0002
5.2 to 6.2	Methyl malonic acid	50	Na^+ or Li^+	5.76	
5.6 to 6.6	MES	50	Na^+ or Li^+	6.27	-0.0110
6.7 to 7.7	Phosphate	50	Na^+	7.20	-0.0028
7.0 to 8.0	HEPES	50	Na^+ or Li^+	7.56	-0.0140
7.8 to 8.8	BICINE	50	Na^+	8.33	-0.0180

[1] *Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002 to 2003.*

Volatile buffer systems

pH range	Buffer system	Counterion	pKa-values for buffering ions[1]
3.3 to 4.3	Formic acid	H^+	3.75
3.3 to 4.3; 4.8 to 5.8	Pyridine/formic acid	$HCOO^-$	3.75; 5.25
3.3 to 4.3; 9.3 to 10.3	Trimethylamine/formic acid	$HCOO^-$	4.75; 9.81
4.3 to 5.8	Pyridine/acetic acid	CH_3COO^-	4.75; 5.25
4.3 to 5.3; 9.3 to 10.3	Trimethylamine/acetic acid	CH_3COO^-	4.75; 9.81
3.3 to 4.3; 8.8 to 9.8	Ammonia/formic acid	$HCOO^-$	3.75; 9.25
4.3 to 5.3; 8.8 to 9.8	Ammonia/acetic acid	CH_3COO^-	4.75; 9.25
5.9 to 6.9; 9.3 to 10.3	Trimethylamine/carbonate	CO_3^{2-}	6.35; 9.81
5.9 to 6.9; 8.8 to 9.8	Ammonium bicarbonate	HCO_3^-	6.35; 9.25
5.9 to 6.9; 8.8 to 9.8	Ammonium carbonate/ammonia	CO_3^{2-}	6.35; 9.25
5.9 to 6.9; 8.8 to 9.8	Ammonium carbonate	CO_3^{2-}	6.35; 9.25
4.3 to 5.3: 7.2 to 8.2	N-Ethylmorpholine/acetate	$HCOO^-$	4.75; 7.72

[1] *Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002 to 2003.*

Appendix 3
Column packing and preparation

Prepacked columns from GE will ensure reproducible results and the highest performance.

Use small prepacked columns for media scouting and method optimization, to increase efficiency in method development, for example, HiTrap IEX Selection Kit.

Efficient column packing is essential for IEX separation, especially when using gradient elution. A poorly packed column gives rise to poor and uneven flow, band broadening, and loss of resolution. If column packing is required, the following guidelines will apply at all scales of operation:

- With a high binding capacity medium, use short, wide columns (typically 5 to 15 cm bed height) for rapid purification, even at low flow velocity.
- The amount of IEX medium required will depend on the binding capacity of the medium and the amount of sample. Binding capacities for each medium are given in this handbook and supplied with the product instructions. Estimate the amount of medium required to bind the sample of interest and use five times this amount to pack the column. The amount of medium required can be reduced if resolution is satisfactory.
- Once separation parameters have been determined, scale up a purification by increasing the diameter of the column to increase column volume. Avoid increasing the length of the column, if possible, as this will alter separation conditions.

IEX media can be packed in either Tricorn, XK, or HiScale columns available from GE. A step-by-step demonstration of column packing can be seen in *Column Packing — The Movie* (Fig A3.1), available in CD format (see *Ordering information*).

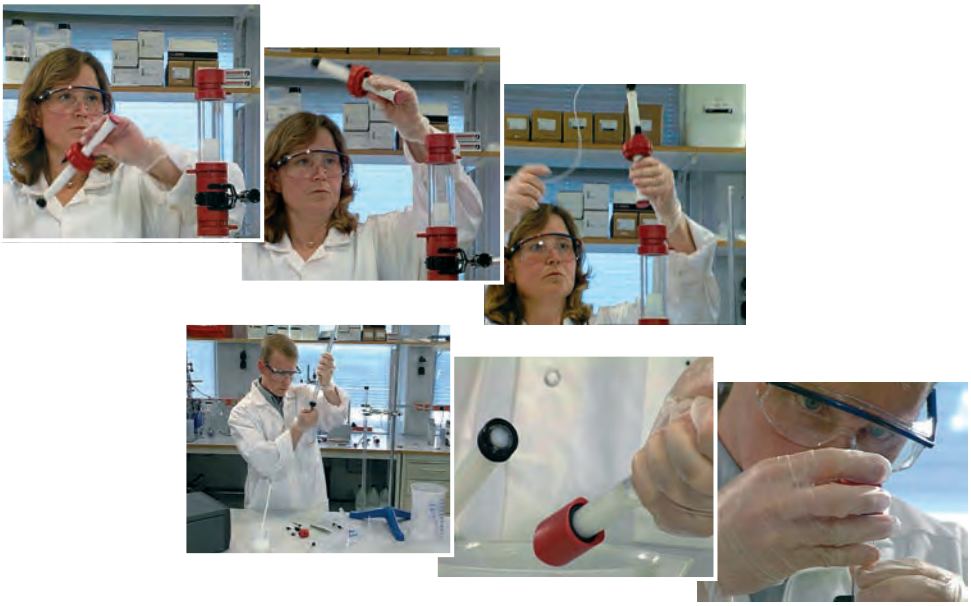


Fig A3.1. *Column Packing — The Movie* provides a step-by-step demonstration of column packing.

1. Equilibrate all materials to the temperature at which the separation will be performed.
2. Eliminate air by flushing column end pieces with the recommended buffer. Ensure no air is trapped under the column net. Close column outlet leaving 1 to 2 cm of buffer in the column.
3. Gently resuspend the medium.

Note that IEX media from GE are supplied ready to use. Decanting of fines that could clog the column is unnecessary.

Avoid using magnetic stirrers since they can damage the matrix.

4. Estimate the amount of slurry (resuspended medium) required on the basis of the recommendations supplied.
5. Pour the required volume of slurry into the column. Pouring down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
6. Immediately fill the column with buffer.
7. Mount the column top piece and connect to a pump.
8. Open the column outlet and set the pump to the desired flow rate.

When slurry volume is greater than the total volume of the column, connect a second glass column to act as a reservoir (see *Ordering information* for details). This ensures that the slurry has a constant diameter during packing, minimizing turbulence and improving column packing conditions.

If the recommended flow rate cannot be obtained, use the maximum flow rate the pump can deliver.

9. Maintain the packing flow rate for at least 3 CV after a constant bed height is obtained. Mark the bed height on the column.

Do not exceed 75% of the packing flow rate during any purification.

10. Stop the pump and close the column outlet. Remove the top piece and carefully fill the rest of the column with buffer to form an upward meniscus at the top.
11. Insert the adapter into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adapter slowly down the column (the outlet of the adaptor should be open) until the mark is reached. Lock the adapter in position.
13. Connect the column to the pump and begin equilibration. Reposition the adapter if necessary.

The medium must be thoroughly washed to remove the storage solution, usually 20% ethanol. Residual ethanol can interfere with subsequent procedures.

Many media equilibrated with sterile phosphate-buffered saline containing an antimicrobial agent may be stored at 4°C for up to 1 mo, but always follow the specific storage instructions supplied with the product.

Column selection

Tricorn, XK, and HiScale columns are fully compatible with the high flow rates achievable with modern media and a broad range of column dimensions are available. Columns most suitable for packing IEX media are listed under the column packing section for each IEX medium (Chapter 3). In most cases the capacity of the IEX medium and the amount of sample to be purified will determine the column size required.

Column packing and efficiency

Column efficiency is expressed as the number of theoretical plates per meter chromatography bed (N) or as H (height equivalent to a theoretical plate, HETP), which is the bed length (L) divided by the plate number. Column efficiency is related to the band broadening that can occur on a column and can be calculated from the expression:

$$N = 5.54 \times \left(\frac{V_R}{W_h} \right)^2$$

V_R = volume eluted from the start of sample application to the peak maximum

w_h = peak width measured as the width of the recorded peak at half of the peak height

H is calculated from the expression:

$$H = \frac{L}{N}$$

L = height of packed bed.

Measurements of V_R and w_h can be made in distance (mm) or volume (ml) but both parameters must be expressed in the same unit.

Column performance should be checked at regular intervals by injecting acetone to determine column efficiency (N) and peak symmetry (asymmetry factor, A_s). Since the observed value for N depends on experimental factors such as flow rate and sample loading, comparisons must be made under identical conditions. In IEX, efficiency is measured under isocratic conditions by injecting acetone (which does not interact with the medium) and measuring the eluted peak as shown in Figure A3.2.

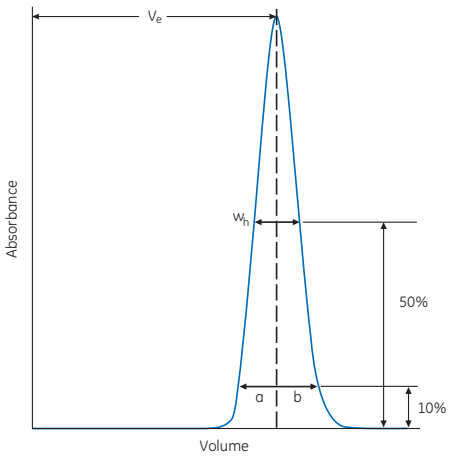


Fig A3.2. Measurements taken to calculate column efficiency.

As a general rule, a good H value is about two to three times the average particle diameter of the medium being packed. For a 90 µm particle, this means an H value of 0.018 to 0.027 cm.

The asymmetry factor (A_s) is expressed as:

$$A_s = \frac{b}{a}$$

where

a = First half peak width at 10% of peak height

b = Second half peak width at 10% of peak height

A_s should be as close as possible to 1.0. A reasonable A_s value for a short column as used in IEX is 0.80 to 1.80.

An extensive leading edge is usually a sign that the medium is packed too tightly and extensive tailing is usually a sign that the medium is packed too loosely.

Run at least two column volumes of buffer through a newly packed column to ensure that the medium is equilibrated with start buffer. Use pH monitoring to check the pH of the eluent.

Appendix 4
Selection of purification equipment

Simple IEX, such as elution by a step-gradient, can be performed using a syringe or peristaltic pump with prepacked HiTrap columns. A chromatography system is required when reproducible results are important and when manual purification becomes too time-consuming and inefficient. This can be the case when large sample volumes are handled, or when there are many different samples to be purified. The progress of the purification can be monitored automatically and high-resolution separations with accurately controlled linear-gradient elution can be performed.

Table A4.1 lists the standard ÄKTA system configurations for currently available systems, see also *ÄKTA Laboratory-scale Systems: Instrument Management Handbook*, 29010831. Table 4.2 on the next page provides a summary of prepacked IEX columns for use with ÄKTA systems.

Table A4.1. Ways of working with standard ÄKTA chromatography systems

Way of working		ÄKTA start	ÄKTAprime plus	ÄKTAxpress	ÄKTA pure		ÄKTA avant
Simple, one-step desalting, buffer exchange	Research →	•	•	•	•	Process development →	•
Automated and reproducible protein purification using all common techniques including support for gradient elution		•	•	•	•		•
Software compatible with regulatory requirements, e.g., good laboratory practice (GLP)				•	•		•
Method development and optimization using design of experiments (DoE)			o	•			•
Automatic buffer preparation including pH scouting				•	o		•
Automatic medium or column scouting			o	•	o		•
Automatic, multistep purification			o	•	o		o
Scale-up, process development			o	o	o		•
Flow rate (ml/min)		0.5 to 5.0	0.1 to 50.0	0.1 to 65.0	0.001 to 25.0 (ÄKTA pure 25)/ 0.01 to 150 (ÄKTA pure 150)		0.001 to 25/0.01 to 150
Max. operating pressure (MPa)		0.5	1	3	20/5		20/5
Software[1] for system control and data handling		UNICORN™ start	PrimeView™[2]	UNICORN 5	UNICORN 6 or later		UNICORN 6 or later

[1] *A specific software version might be needed for the chosen system. See the web page for each respective system at www.gelifesciences.com/AKTA.*

[2] *With PrimeView, you can monitor results and evaluate data but not create methods nor control the system.*

• = *included*
o = *optional*

Table A4.2. Summary of prepacked columns for IEX using ÄKTA systems

Chromatography technique	Base matrix	HiTrap	HiScreen	HiPrep	RESOURCE	Tricorn (GL/PE)	Precision Columns (PC)[7]
		Easy to use with a syringe, peristaltic pump, or chromatography system	Optimized for method and process development	Convenient scale-up Preparative size exclusion chromatography	Fast with good resolution	High quality and high resolution	Micro-purification and analysis
Ion exchange	Sepharose HP, Sepharose FF	✓	✓	✓			
	Capto, Capto ImpRes	✓	✓				
	Capto ImpAct	✓	✓				
	MonoBeads					✓	✓
	MiniBeads					✓	✓
	SOURCE				✓	✓	
System compatibility		ÄKTA pure ÄKTA start ÄKTAxpress ÄKTAprime plus ÄKTApurifier[2] ÄKTAexplorer[3] ÄKTAFPLC[4]	ÄKTA avant ÄKTA pure 150 ÄKTApurifier 100[2] ÄKTAexplorer 100[3]	ÄKTA pure ÄKTA start[5] ÄKTA avant ÄKTAxpress ÄKTAprime plus ÄKTApurifier[2] ÄKTAexplorer[3] ÄKTAFPLC[4]	ÄKTA pure ÄKTAmicro[6] ÄKTApurifier 10[2] ÄKTAexplorer 10[3] ÄKTAFPLC[4]	ÄKTA pure 25 ÄKTAxpress ÄKTAmicro[6] ÄKTApurifier 10[2] ÄKTAexplorer 10[3] ÄKTAFPLC[4]	ÄKTAmicro[6]

1 *Sample volume.*
2 *ÄKTApurifier has been discontinued and replaced by ÄKTA pure.*
3 *ÄKTAexplorer has been discontinued and replaced by ÄKTA avant.*
4 *ÄKTAFPLC has been discontinued and replaced by ÄKTA pure 25.*
5 *HiPrep 26/60 can be used but is not optimal.*
6 *ÄKTAmicro has been discontinued and replaced by ÄKTA pure 25 with microgram-scale purification flow path.*
7 *Precision Columns (PC) provide excellent results when used in combination with HPLC systems.*

Appendix 5
Converting from flow velocity to volumetric flow rates

It is convenient when comparing results for columns of different sizes to express flow as flow velocity (cm/h). However, flow is usually measured in volumetric flow rate (ml/min). To convert between flow velocity and volumetric flow rate use one of the formulae below.

From flow velocity (cm/h) to volumetric flow rate (ml/min)

$$\text{Volumetric flow rate (ml/min)} = \frac{\text{Flow velocity (cm/h)}}{60} \times \text{column cross sectional area (cm}^2\text{)}$$

$$= \frac{Y}{60} \times \frac{\pi \times d^2}{4}$$

where

Y = flow velocity in cm/h
d = column inner diameter in cm

Example:
What is the volumetric flow rate in an XK 16/70 column (i.d. 1.6 cm) when the flow velocity is 150 cm/h?

Y = flow velocity = 150 cm/h
d = inner diameter of the column = 1.6 cm

$$\text{Volumetric flow rate} = \frac{150 \times \pi \times 1.6 \times 1.6}{60 \times 4} \text{ ml/min}$$

$$= 5.03 \text{ ml/min}$$

From volumetric flow rate (ml/min) to flow velocity (cm/h)

$$\text{Flow velocity (cm/h)} = \frac{\text{Volumetric flow rate (ml/min)} \times 60}{\text{column cross sectional area (cm}^2\text{)}}$$

$$= Z \times 60 \times \frac{4}{\pi \times d^2}$$

where

Z = volumetric flow rate in ml/min
d = column inner diameter in cm

Example:

What is the linear flow in a Tricorn 5/50 column (i.d. 0.5 cm) when the volumetric flow rate is 1 ml/min?

Z = volumetric flow rate = 1 ml/min
d = column inner diameter = 0.5 cm

$$\text{Flow velocity} = 1 \times 60 \times \frac{4}{\pi \times 0.5 \times 0.5} \text{ cm/h}$$

From volumetric flow rate (ml/min) to using a syringe

1 ml/min = approximately 30 drops/min on a HiTrap 1 ml column
5 ml/min = approximately 120 drops/min on a HiTrap 5 ml column

Appendix 6
Conversion data: proteins, column pressures

Proteins

Mass (g/mol)	1 µg	1 nmol
10 000	100 pmol; 6×10^{13} molecules	10 µg
50 000	20 pmol; 1.2×10^{13} molecules	50 µg
100 000	10 pmol; 6.0×10^{12} molecules	100 µg
150 000	6.7 pmol; 4.0×10^{12} molecules	150 µg
1 kb of DNA	= 333 amino acids of coding capacity	
	= 37 000 g/mol	
270 bp DNA	= 10 000 g/mol	
1.35 kb DNA	= 50 000 g/mol	
2.70 kb DNA	= 100 000 g/mol	

Average molecular weight of an amino acid = 120 g/mol.

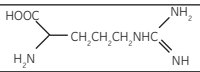
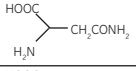
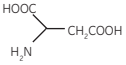
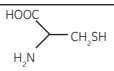
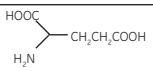
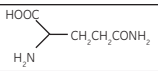
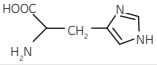
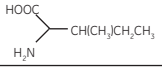
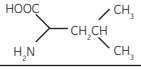
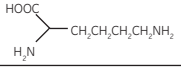
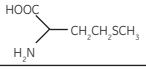
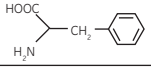
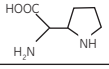
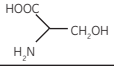
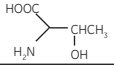
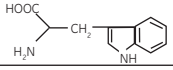
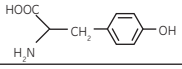
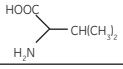
Protein	A_{280} for 1 mg/ml
IgG	1.35
IgM	1.20
IgA	1.30
Protein A	0.17
Avidin	1.50
Streptavidin	3.40
Bovine serum albumin	0.70

Column pressures

The maximum pressure drop over the packed bed refers to the pressure above which the column contents might begin to compress.

Pressure units may be expressed in megaPascal (MPa), bar, or pounds per square inch (psi) and can be converted as follows: 1 MPa = 10 bar = 145 psi

Appendix 7
Table of amino acids

Amino acid	Three-letter code	Single-letter code	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic acid	Asp	D	
Cysteine	Cys	C	
Glutamic acid	Glu	E	
Glutamine	Gln	Q	
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	

Formula	M_r	Middle unit residue (-$H_2$0) Formula	M_r	Charge at pH 6.0 to 7.0	Hydrophobic (nonpolar)	Uncharged (polar)	Hydrophilic (polar)
$C_3H_7NO_2$	89.1	C_3H_5NO	71.1	Neutral	•		
$C_6H_{14}N_4O_2$	174.2	$C_6H_{12}N_4O$	156.2	Basic (+ve)			•
$C_4H_8N_2O_3$	132.1	$C_4H_6N_2O_2$	114.1	Neutral		•	
$C_4H_7NO_4$	133.1	$C_4H_5NO_3$	115.1	Acidic(-ve)			•
$C_3H_7NO_2S$	121.2	C_3H_5NOS	103.2	Neutral		•	
$C_5H_9NO_4$	147.1	$C_5H_7NO_3$	129.1	Acidic (-ve)			•
$C_5H_{10}N_2O_3$	146.1	$C_5H_8N_2O_2$	128.1	Neutral		•	
$C_2H_5NO_2$	75.1	C_2H_3NO	57.1	Neutral		•	
$C_6H_9N_3O_2$	155.2	C6H_7N_3O	137.2	Basic (+ve)			•
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	•		
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	•		
$C_6H_{14}N_2O_2$	146.2	$C_6H_{12}N_2O$	128.2	Basic (+ve)			•
$C_5H_{11}NO_2S$	149.2	C_5H_9NOS	131.2	Neutral	•		
$C_9H_{11}NO_2$	165.2	C_9H_9NO	147.2	Neutral	•		
$C_5H_9NO_2$	115.1	C_5H_7NO	97.1	Neutral	•		
$C_3H_7NO_3$	105.1	$C_3H_5NO_2$	87.1	Neutral		•	
$C_4H_9NO_3$	119.1	$C_4H_7NO_2$	101.1	Neutral		•	
$C_{11}H_{12}N_2O_2$	204.2	$C_{11}H_{10}N_2O$	186.2	Neutral	•		
$C_9H_{11}NO_3$	181.2	$C_9H_9NO_2$	163.2	Neutral		•	
$C_5H_{11}NO_2$	117.1	C_5H_9NO	99.1	Neutral	•		

Appendix 8
Analytical assays during purification

Analytical assays are essential to follow the progress of purification. They are used to assess the effectiveness of each step in terms of yield, biological activity, and recovery as well as to help during optimization of experimental conditions. The importance of a reliable assay for the target molecule cannot be overemphasized.

When testing chromatographic fractions, ensure that the buffers used for purification do not interfere with the assay.

Total protein determination

Lowry or Bradford assays are used most frequently to determine the total protein content. The Bradford assay is particularly suited to samples where there is a high lipid content that can interfere with the Lowry assay.

Purity determination

Purity is most often estimated by SDS-PAGE. Alternatively, isoelectric focusing, capillary electrophoresis, reversed phase chromatography, or mass spectrometry may be used.

SDS-PAGE analysis

The general steps involved in SDS-PAGE analysis are summarized below.

1. Prepare samples by mixing with equal volume of 2x SDS loading buffer
2. Vortex briefly and heat for 5 min at 90°C to 100°C.
3. Load the samples and, optionally, a MW marker onto a SDS-polyacrylamide gel.
4. Run the gel.
5. Stain the gel with Coomassie Blue (Coomassie Blue Tablets, PhastGel Blue R-350) or silver (PlusOne Silver Staining Kit, Protein).

The percentage of acrylamide in the SDS gel should be selected according to the expected molecular weight of the protein of interest (see Table A8.1).

Table A8.1. Percentage of acrylamide used in SDS gels for proteins of different molecular weights

Acrylamide in resolving gel (%)		Mol. weight range
Homogeneous:	5	36 000 to 200 000
	7.5	24 000 to 200 000
	10	14 000 to 200 000
	12.5	14 000 to 100 000
	15	14 000 to 60 000[1]
Gradient:	5 to 15	14 000 to 200 000
	5 to 20	10 000 to 200 000
	10 to 20	10 000 to 150 000

[1] *The larger proteins fail to move significantly into the gel.*

The gel is usually stained after electrophoresis in order to make the protein bands visible by, for example, Coomassie Blue or silver staining. A more recent way of making protein visible is by prelabeling the proteins by fluorescent dye (Amersham™ WB Cy™5 dye reagent) before loading the sample in the gel. By doing in this way the gel image can be acquired directly after finished electrophoresis by laser scanner or CCD camera and the result is obtained much faster. This workflow is outlined below.

Protein prelabeling with CyDye™

1. Prepare samples by prelabeling with Amersham WB Cy5 dye reagent.
2. Vortex briefly and heat for 5 min at 90°C to 100°C.
3. Load the samples and, optionally, a MW marker onto a SDS-polyacrylamide gel.
4. Run the gel and proceed directly to image capture.

For information and advice on electrophoresis techniques, refer to the handbook *2-D Electrophoresis, Principles and Methods*, 80642960. For information on the Amersham WB system and accessories including Amersham WB Cy5 prelabeling reagents, visit www.gelifesciences.com/westernblotting.

Functional assays

Immunospecific interactions have enabled the development of many alternative assay systems for the assessment of active concentration of target molecules.

- Western blot analysis is used to confirm protein identity and quantitate the level of target molecule.

1. Separate the protein samples by SDS-PAGE.
2. Transfer the separated proteins from the gel to an appropriate membrane, depending on the choice of detection reagents. Amersham Protran™ (NC) or Amersham Hybond™ P (PVDF) membranes are recommended for chemiluminescent detection using Amersham ECL™ start, Amersham ECL, Amersham ECL Prime, or Amersham ECL Select™ Western blotting detection reagents. Amersham Protran Premium (NC) or Amersham Hybond LFP (PVDF) membranes are recommended for fluorescent detection with Amersham ECL Plex™ Western blotting detection system.
3. Develop the membrane with the appropriate specified reagents.

Electrophoresis, protein transfer, and probing may be accomplished using a variety of equipment and reagents. The Amersham WB system is an automated system that can be used for all these steps including software evaluation. For more information, visit www.gelifesciences.com/westernblotting. For further on the basic principles and methods used in Western blotting, refer to the *Western Blotting Handbook*, 28999897 and the instruction manuals supplied with the detection kits.

- ELISAs are most commonly used as activity assays.
- Functional assays using the phenomenon of surface plasmon resonance (SPR) to detect immunospecific interactions (e.g., using Biacore™ systems) enable the determination of active concentration, epitope mapping, and studies of interaction kinetics.

The *Biacore Assay Handbook*, 29019400 gives a general overview of the different types of SPR-based applications. The handbook also provides advice on sample preparation, design, and optimization of different assays.

Detection and assay of tagged proteins

SDS-PAGE, Western blotting, and ELISA can also be applied to the detection and assay of genetically engineered molecules to which a specific tag has been attached. In some cases, an assay based on the properties associated with the tag itself can be developed, for example, the GST Detection Module for enzymatic detection and quantitation of GST-tagged proteins. Further details on the detection and quantitation of GST and $(his)_6$-tagged proteins are available in the *Recombinant Protein Purification Handbook*, 18114275 and the *GST Gene Fusion System Handbook*, 18115758 from GE.

Appendix 9
Storage of biological samples

The advice given here is of a general nature and cannot be applied to every biological sample. Always consider the properties of the specific sample and its intended use before following any of these recommendations.

General recommendations

- Add stabilizing agents, when necessary. Stabilizing agents are often required for storage of purified proteins.
- Serum, culture supernatants, and ascitic fluid should be kept frozen at -20°C or -70°C, in small aliquots.
- Avoid repeated freeze/thawing or freeze drying/redissolving that can reduce biological activity.
- Avoid conditions close to stability limits for example pH or salt concentrations, reducing or chelating agents.
- Keep refrigerated at 4°C in a closed vessel to minimize bacterial growth and protease activity. Above 24 h at 4°C, add a preserving agent if possible (e.g., merthiolate 0.01%).

Sodium azide can interfere with many coupling methods and some biological assays and can be a health hazard. It can be removed by using a desalting column (see Appendix 1, *Sample preparation*).

Specific recommendations for purified proteins

- Store as a precipitate in high concentration of ammonium sulfate, for example 4.0 M.
- Freeze in 50% glycerol, especially suitable for enzymes.
- Avoid the use of preserving agents if the product is to be used for a biological assay. Preserving agents should not be added if *in vivo* experiments are to be performed. Store samples in small aliquots and keep frozen.
- Sterile filter to prolong storage time.
- Add stabilizing agents such as glycerol (5% to 20%) or serum albumin (10 mg/ml) to help maintain biological activity. Remember that any additive will reduce the purity of the protein and might need to be removed at a later stage.
- Avoid repeated freeze/thawing or freeze drying/redissolving that can reduce biological activity.

Certain proteins, including some mouse antibodies of the IgG_3 subclass, should not be stored at 4°C as they precipitate at this temperature. Keep at room temperature in the presence of a preserving agent.

Appendix 10
Column cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure, or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

Reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step varies according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this can affect the column packing and interfere with performance. The cleaning procedure to remove common contaminants is included with each of the media described in in Chapter 3.

Removing precipitated proteins, lipids, hydrophobically bound proteins, or lipoproteins

To remove precipitated proteins

Use the recommended flow rate for cleaning of the media and column, see Chapter 3. When guanidine hydrochloride is used, the flow rate should be lowered to half of this flow rate to avoid overpressure.

1. Wash with 2 CV of 6 M guanidine hydrochloride.
2. Wash immediately with at least 5 CV of buffer at pH 7.0 to 8.0.
3. Rinse with at least 2 CV of distilled water until the UV-baseline and eluent pH are stable.
4. Wash with at least 4 CV of start buffer or storage buffer until pH and conductivity values have reached the required values.

Alternatively,

1. Inject 1 CV of pepsin (1 mg/ml in 500 mM NaCl, 100 mM acetic acid). Leave overnight at room temperature or for 1 h at 37°C.
2. Rinse with at least 2 CV of distilled water until the UV-baseline and the eluent pH are stable.
3. Wash with at least 4 CV of start buffer or storage buffer, until eluent pH and conductivity have reached the required values.

To remove lipids, hydrophobically bound proteins, or lipoproteins

Organic solvents or detergents might be required to completely remove contaminants of this type.

Before using organic solvents, wash the medium with at least 4 CV of distilled water to avoid salts precipitating on the column.

When applying organic solvents or solutions it might be necessary to reduce the flow rate considerably to avoid overpressuring the column.

Use cleaning solutions such as up to 30% isopropanol, up to 100% methanol, up to 100% acetonitrile, up to 2 M NaOH, up to 75% acetic acid, up to 100% ethanol, ionic or nonionic detergents.

When cleaning larger columns, allow a contact time of 1 to 2 h for any solution that is used as an initial cleaning step.

Always check for solvent compatibility in the instructions supplied with the medium or column.

Avoid anionic detergents with Q, DEAE, and ANX charged groups. Avoid cationic detergents with S, SP, and CM charged groups.

Extended cleaning procedures

Use the recommended flow rate and cleaning procedure for the media and column, see Chapter 3. If this is not sufficient, extended cleaning procedures can be tested. When organic solvents, such as 30% isopropanol or 70% ethanol are used, the flow rate should be lowered to half of this flow rate to avoid overpressure.

1. Wash with 4 CV of up to 70% ethanol or 30% isopropanol.
2. Rinse with at least 2 CV of distilled water until the UV-baseline and eluent pH are stable.
3. Wash immediately with 3 CV of start buffer.

Alternatively,

1. Wash with 2 CV of detergent in a basic or acidic solution, for example, 0.1% to 0.5% nonionic detergent in 100 mM acetic acid.
2. Rinse with 5 CV 70% ethanol to remove residual detergent.
3. Rinse with at least 2 CV of distilled water until the UV-baseline and the eluent pH are stable.
4. Wash with 3 CV of start buffer.

Appendix 11
Media selection

Using prepacked small columns such as HiTrap during the early stages of development saves time, solvents and sample. HiTrap IEX Selection Kit allows quick and efficient screening for the most suitable charge group and enables development of the basic method. The following are descriptions of different screening methods for selection of media and optimal conditions.

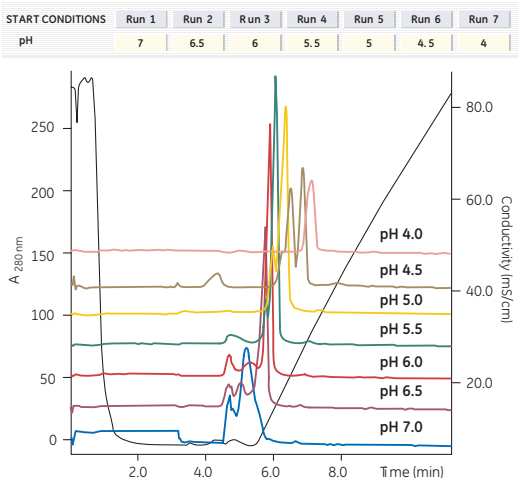
Selection of media for automated purification

Users of ÄKTA systems with automatic buffer preparation functionality can select from a range of buffer recipes to test different media over a range of pH values and other elution conditions. See *ÄKTA Laboratory-scale Chromatography Systems: Instrument Management Handbook*, 29010831 for details of how to vary flow rate and gradient slope in order to optimize the separation.

Note that the condition of the sample is very important in order to achieve the most effective separations. Samples should preferably have the same conditions as the start buffer (see *Buffer exchange and desalting* in Appendix 1 for details). When working with small volumes during screening and scouting, it might be sufficient to dilute the sample in start buffer in order to lower the ionic strength and adjust the pH to a value similar to that of the start buffer.

1. Scout for optimum pH by testing a range of pH values within which the proteins of interest are known to be stable. If the pI of the target protein is known, then begin with a narrower pH range, for example, 0.5 to 1.0 pH unit away from the pI. Typical results from an automatic pH scouting run are shown in Figure A11.1.
2. If required, scout for optimum selectivity (testing strong or weak exchangers) using automatic media scouting.
3. Scout for the steepest gradient that gives acceptable resolution at the selected pH.
4. Scout for the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
5. Scout for the maximum sample load that can be applied while maintaining satisfactory resolution. In general, loading 20% to 30% of the total binding capacity of the column gives optimal resolution with gradient elution.

Reduce separation time and buffer consumption by transfering to a step elution when optimized separation conditions have been established. Sample loads can often be increased when using a step elution.



Column: RESOURCE S, 6 ml
Sample: Fab fraction from HIC separation, 20 ml
Eluents: Automatic BufferPrep with
60 mM sodium acetate,
30 mM sodium phosphate,
30 mM sodium formate
100 mM HCl and 2 M NaCl
Gradient: 20 column volumes, to 1 M NaCl
Flow rate: 60 ml/min
Curves: A_{280nm}, from top:
pH 4.0; 4.5; 5.0; 5.5; 6.0; 6.5; 7.0

Fig A11.1. Automatic pH scouting on an ÄKTA system.

Selection of media for manual purification

HiTrap columns are well-suited to manual media screening, method development, and method optimization since they can be used with a syringe or peristaltic pump as well as an automated chromatography system.

Note that the condition of the sample is very important to achieve the most effective separations. Samples should preferably have the same conditions the start buffer (see Appendix 1, *Sample preparation* for details). When working with small volumes during screening it might be sufficient to dilute the sample in start buffer in order to lower the ionic strength and adjust the pH to a value similar to that of the start buffer.

Scout for optimum pH by testing a range of pH values within which the proteins of interest are known to be stable. If the pI of the target protein is known, then begin with a narrower pH range, for example, 0.5 to 1.0 pH unit away from the pI. The methods here are optimized for use with 1 ml HiTrap columns and should be adjusted if other column volumes are used.

Screening for IEX medium and pH conditions

1. Start buffers: set up a series of buffers with pH values in the range 4.0 to 8.0 (SP, CM) or 5.0 to 9.0 (Q, DEAE, ANX) and with 0.5 to 1.0 pH unit intervals between each buffer. See Appendix 2 for recommended buffers.
2. Elution buffers: set up a second series of buffers with the same pH values, but including 1 M NaCl.
3. Equilibrate the column (s) with 5 ml start buffer at 1 ml/min. Wash with 5 ml elution buffer.
4. Re-equilibrate with 5 to 10 ml start buffer.
5. Adjust the sample to the pH of the start buffer and apply a known amount of the sample at 1 ml/min. Collect eluate.
6. Wash with at least 5 ml of start buffer or until no material appears in eluent. Collect eluate.
7. Elute bound material with elution buffer (3 to 5 ml is usually sufficient, but other volumes might be required dependent on the exact experimental conditions). Collect eluate.

7. Elute bound material with elution buffer (3 to 5 ml is usually sufficient, but other volumes might be required dependent on the exact experimental conditions). Collect eluate.
8. Analyze all eluates (for example by an activity assay) and determine purity and the amount bound to the column.
9. Perform steps 3 to 8 for the next buffer pH.
10. Select medium and pH: the most suitable pH should allow the protein(s) of interest to bind, but should be as close to their point of release as possible.

Screening for ionic strength conditions

1. Using the selected medium, start buffer and pH from the previous protocol, set up a series of elution buffers at the same pH, but vary the salt concentration from 0 to 500 mM with intervals of 50 to 100 mM salt between each buffer.
2. Repeat steps 3 to 8 from the previous protocol for each salt concentration.
3. Determine the maximum ionic strength which permits binding of the protein(s) of interest and the minimum ionic strength required for complete elution.

Further optimization

1. If gradient making equipment is available, determine the steepest gradient that gives acceptable resolution at the selected pH. Begin with a gradient of 10 CV over an ionic strength range based on the maximum and minimum values determined when screening. Alternatively, begin with a gradient of 0% to 50% elution buffer that contains 1 M NaCl and a gradient volume of 10 to 20 CV.
2. Determine the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
3. Determine the maximum sample load that can be applied while maintaining satisfactory resolution. In general, loading 20% to 30% of the total binding capacity of the column gives optimal resolution with gradient elution. Sample loads can often be increased if resolution is satisfactory or when using a step elution.

Using PD-10 columns for media selection and method development

If an assay is available to detect the target protein(s), PD-10 columns can be packed with various media and used to find the most suitable separation conditions. With basic information on the requirements for pH and ionic strength, a suitable column can be packed in order to begin optimization.

Note that the condition of the sample is very important in order to achieve the most effective separations. Samples should preferably have the same conditions as the start buffer (see Appendix 1, *Sample preparation* for details). When working with small volumes during screening and scouting, it might be sufficient to dilute the sample in start buffer in order to lower the ionic strength and adjust the pH to a value similar to that of the start buffer.

pH selection

1. Set up a series of 10 × PD-10 columns for each medium to be tested and thoroughly resuspend the medium in its storage solution.
2. Pour medium slurry containing 5 ml medium into the PD-10 column, allowing the medium to settle as the column fills. Do not allow the column to dry out.
3. Equilibrate each column to a different pH by washing (5 × 5 ml) with buffer (500 mM) using buffers between pH 5.0 to 9.0 for anion exchangers or pH 4.0 to 8.0 for cation exchangers and with 0.5 pH unit intervals between columns (see Appendix 2 for buffer recommendations).
4. Equilibrate each column at a lower ionic strength: wash with 5 × 5 ml of buffer (20 to 50 mM) at the same pH.
5. Load a known constant amount of sample to each column while collecting the eluent.
8. Assay the eluent for the protein of interest. The most suitable medium and pH should allow the protein to bind (protein is absent from the eluent), but should be as close to the point of release as possible (the first pH at which the protein appears in the eluent).

Ionic strength selection

1. Set up a series of 10 × PD-10 columns, each containing 5 ml of the chosen IEX medium.
2. Equilibrate the column by washing (5 × 5 ml) with buffer (500 mM) at the selected starting pH.
3. Equilibrate the columns at different ionic strengths, but constant pH, ranging from 10 to 300 mM NaCl by washing (5 × 5 ml). Intervals of 50 mM NaCl are sufficient.
4. Apply sample while collecting the eluent.
5. Assay the eluent to determine the maximum ionic strength which permits binding of the target protein and the minimum ionic strength required for complete elution. The highest ionic strength which permits binding and the lowest ionic strength for elution are used as start and elution buffers, respectively, during subsequent gradient elution.

Related literature

	Code number
Purification handbooks	
Affinity Chromatography	18102229
Antibody Purification	18103746
Hydrophobic Interaction and Reversed Phase Chromatography	11001269
Multimodal Chromatography	29054808
Protein Sample Preparation	28988741
Purifying Challenging Proteins	28909531
Recombinant Protein Purification	18114275
Size Exclusion Chromatography	18102218
Strategies for Protein Purification	28983331
ÄKTA Laboratory-scale Chromatography Systems	29010831
Protein analysis handbooks	
Biacore Assay	29019400
Biacore Sensor Surface	BR100571
Western Blotting	28999897
Selection guides and multimedia	
Ion exchange columns and media, Selection guide	18112731
Prepacked chromatography columns for ÄKTA systems, Selection guide	28931778
Column Packing - The Movie, CD	18116533

Ordering information

Ion exchange

Product	Quantity	Code number
MiniBeads		
Prepacked columns		
Mini Q PC 3.2/3	1 × 0.24 ml	17068601
Mini S PC 3.2/3	1 × 0.24 ml	17068701
Mini Q 4.6/50 PE	1 × 0.8 ml	17517701
Mini S 4.6/50 PE	1 × 0.8 ml	17517801
MonoBeads		
Prepacked columns		
Mono Q 5/50 GL	1 × 1 ml	17516601
Mono Q 10/100 GL	1 × 8 ml	17516701
Mono Q 4.6/100 PE	1 × 1.7 ml	17517901
Mono Q HR 16/10	1 × 20 ml	17050601
Mono S 5/50 GL	1 × 1 ml	17516801
Mono S 10/100 GL	1 × 8 ml	17516901
Mono S 4.6/100 PE	1 × 1.7 ml	17518001
Mono S HR 16/10	1 × 20 ml	17050701
SOURCE 15		
Chromatography media packs		
SOURCE 15Q	10 ml	17094720
SOURCE 15Q	50 ml	17094701
SOURCE 15Q	200 ml	17094705
SOURCE 15S	10 ml	17094410
SOURCE 15S	50 ml	17094401
SOURCE 15S	200 ml	17094405
Prepacked columns		
RESOURCE Q	1 × 1 ml	17117701
RESOURCE Q	1 × 6 ml	17117901
SOURCE 15Q 4.6/100 PE	1 × 1.7 ml	17518101
RESOURCE S	1 × 1 ml	17117801
RESOURCE S	1 × 6 ml	17118001
SOURCE 15S 4.6/100 PE	1 × 1.7 ml	17518201

Product	Quantity	Code number
SOURCE 30		
Chromatography media packs		
SOURCE 30Q	10 ml	17127510
SOURCE 30Q	50 ml	17127501
SOURCE 30Q	200 ml	17127505
SOURCE 30S	10 ml	17127320
SOURCE 30S	50 ml	17127301
SOURCE 30S	200 ml	17127302
Sepharose High Performance		
Chromatography media packs		
SP Sepharose High Performance	75 ml	17108701
Q Sepharose High Performance	75 ml	17101401
Prepacked columns		
HiTrap Q HP	1 × 1 ml	29051325
HiTrap Q HP	5 × 1 ml	17115301
HiTrap Q HP	5 × 5 ml	17115401
HiPrep Q HP 16/10	1 × 20 ml	29018182
HiTrap SP HP	1 × 1 ml	29051324
HiTrap SP HP	5 × 1 ml	17115101
HiTrap SP HP	5 × 5 ml	17115201
HiScreen SP HP	1 × 4.7 ml	28950515
HiPrep SP HP 16/10	1 × 20 ml	29018183
Sepharose Fast Flow		
Chromatography media packs		
Q Sepharose Fast Flow	25 ml	17051010
Q Sepharose Fast Flow	300 ml	17051001
SP Sepharose Fast Flow	25 ml	17072910
SP Sepharose Fast Flow	300 ml	17072901
DEAE Sepharose Fast Flow	25 ml	17070910
DEAE Sepharose Fast Flow	500 ml	17070901
CM Sepharose Fast Flow	25 ml	17071910
CM Sepharose Fast Flow	500 ml	17071901
ANX Sepharose 4 Fast Flow (high sub)	25 ml	17128710
ANX Sepharose 4 Fast Flow (high sub)	500 ml	17128701
Prepacked columns		
HiTrap IEX Selection Kit	7 × 1 ml	17600233
Kit contains seven HiTrap columns prepacked with Fast Flow (FF) media: Q Sepharose FF, DEAE Sepharose FF, SP Sepharose FF, CM Sepharose FF, ANX Sepharose FF(high sub), Q Sepharose XL, and SP Sepharose XL		
HiTrap Q FF	5 × 1 ml	17505301
HiTrap Q FF	5 × 5 ml	17515601
HiPrep Q FF 16/10	1 × 20 ml	28936543

Product	Quantity	Code number
HiTrap SP FF	5 × 1 ml	17505401
HiTrap SP FF	5 × 5 ml	17515701
HiPrep SP FF 16/10	1 × 20 ml	28936544
HiTrap DEAE FF	5 × 1 ml	17505501
HiTrap DEAE FF	5 × 5 ml	17515401
HiPrep DEAE FF 16/10	1 × 20 ml	28936544
HiTrap CM FF	5 × 1 ml	17505601
HiTrap CM FF	5 × 5 ml	17515501
HiPrep CM FF 16/10	1 × 20 ml	28936542
HiTrap ANX FF (high sub)	5 × 1 ml	17516201
HiTrap ANX FF (high sub)	5 × 5 ml	17516301
Sepharose XL		
Chromatography media packs		
Q Sepharose XL	300 ml	17507201
SP Sepharose XL	300 ml	17507301
Prepacked columns		
HiTrap Q XL	5 × 1 ml	17515801
HiTrap Q XL	5 × 5 ml	17515901
HiPrep Q XL 16/10	1 × 20 ml	28936538
HiTrap SP XL	5 × 1 ml	17516001
HiTrap SP XL	5 × 5 ml	17516101
HiPrep SP XL 16/10	1 × 20 ml	28936540
Sepharose Big Beads		
Chromatography media packs		
Q Sepharose Big Beads	1 l	17098903
SP Sepharose Big Beads	1 l	17065703
Capto		
Chromatography media packs		
Capto Q	25 ml	17531610
Capto Q	100 ml	17531602
Capto S	25 ml	17544110
Capto S	100 ml	17544101
Capto DEAE	25 ml	17544310
Capto DEAE	100 ml	17544301
Prepacked columns		
HiTrap Capto IEX Selection Kit *Contains five HiTrap columns prepacked with: Capto Q, Capto S, Capto DEAE, Capto MMC, Capto adhere*	5 × 1 ml	28934388
HiTrap Capto Q	5 × 1 ml	11001302
HiTrap Capto Q	5 × 5 ml	11001303
HiScreen Capto Q	1 × 4.7 ml	28926978

Product	Quantity	Code number
HiTrap Capto S	5 × 1 ml	17544122
HiTrap Capto S	5 × 5 ml	17544123
HiScreen Capto S	1 × 4.7 ml	28926979
HiTrap Capto DEAE	5 × 1 ml	28916537
HiTrap Capto DEAE	5 × 5 ml	28916540
HiScreen Capto DEAE	1 × 4.7 ml	28926982
Capto ImpRes		
Chromatography media packs		
Capto Q ImpRes	25 ml	17547010
Capto Q ImpRes	100 ml	17547002
Capto SP ImpRes	25 ml	17546810
Capto SP ImpRes	100 ml	17546802
Prepacked columns		
HiTrap Capto Q ImpRes	5 × 1 ml	17547051
HiTrap Capto Q ImpRes	5 × 5 ml	17547055
HiScreen Capto Q ImpRes	1 × 4.7 ml	17547015
HiTrap Capto SP ImpRes	5 × 1 ml	17546851
HiTrap Capto SP ImpRes	5 × 5 ml	17546855
HiScreen Capto SP ImpRes	4.7 ml	17546815
Capto ImpAct		
Chromatography media packs		
Capto S ImpAct	25 ml	17371701
Capto S ImpAct	100 ml	17371702
Prepacked columns		
HiTrap Capto S ImpAct	5 × 1 ml	17371751
HiTrap Capto S ImpAct	5 × 5 ml	17371755
HiScreen Capto S ImpAct	1 × 4.7 ml	17371747

SOURCE, Sepharose High Performance, Sepharose Fast Flow, Sepharose XL, Sepharose Big Beads, and Capto are all available as BioProcess media for large-scale production. Visit www.gelifesciences.com/bioprocess

Prepacked desalting columns

HiTrap Desalting	1 × 5 ml	29048684
HiTrap Desalting	5 × 5 ml	17140801
HiPrep 26/10 Desalting	1 × 53 ml	17508701
HiPrep 26/10 Desalting	4 × 53 ml	17508702
PD-10 Desalting Column	30	17085101

Product	Quantity	Code number
Empty columns		
Tricorn 10/100	1	18116315
XK 16/20	1	18877301
XK 26/20	1	18100072
XK 50/20	1	18100071
Empty Disposable PD-10 Desalting columns	50/pk	17043501
LabMate PD-10 Buffer Reservoir	1	18321603
Acessories and spare parts		
Packing Connector XK 16	1	18115344
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Packing equipment 10/100 (Tricorn)	1	18115325
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Detalle del pago

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