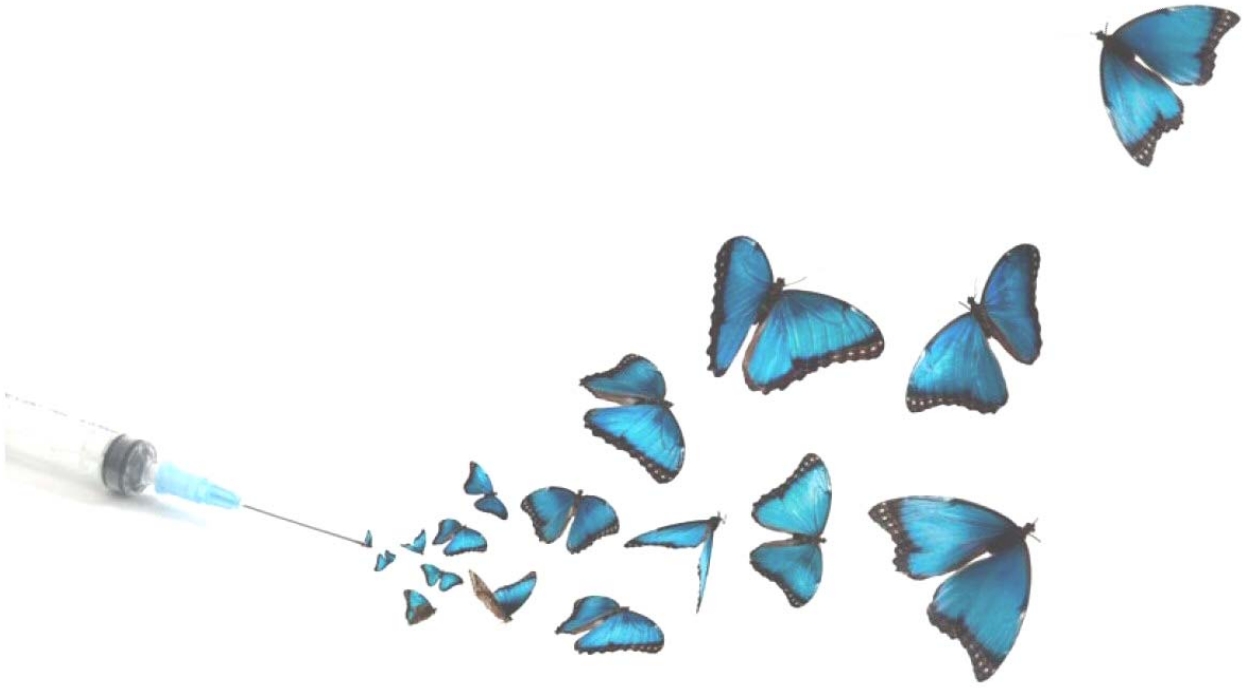
Purification Strategies

纯化策略



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Content

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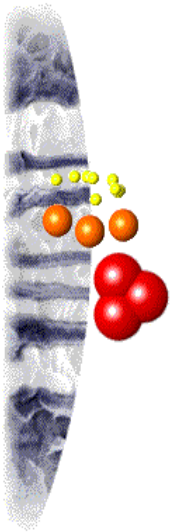
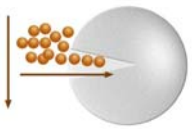
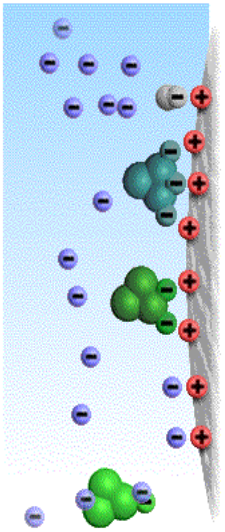
Content

Principles of operation for chromatography techniques 层析原理

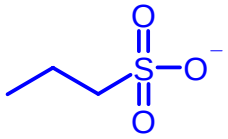


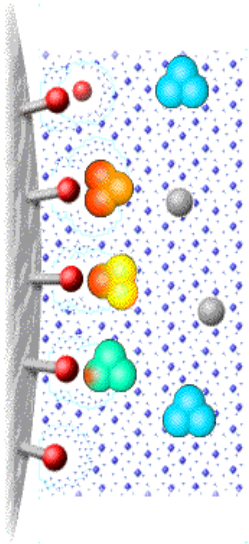
Gel Filtration
分子筛



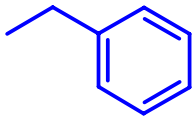


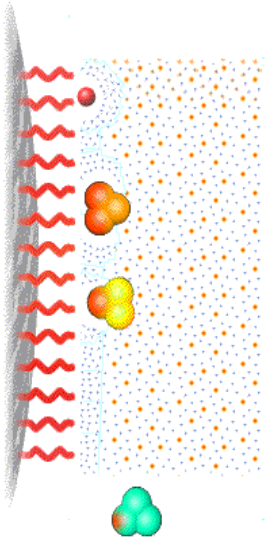
Ion Exchange
离子交换



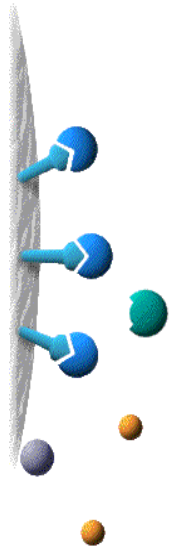


Hydrophobic interaction
疏水层析





Reversed phase
反相



Affinity
亲和

Question

How to use these separation technologies to perform a SUCCESSFUL purification ?

如何运用各种分离纯化技术来实现**成功**的分离纯化？

Content

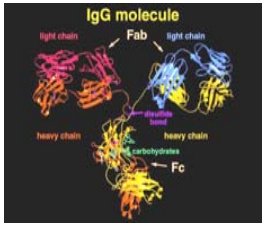
目标:开发有效的蛋白质分离纯化工艺

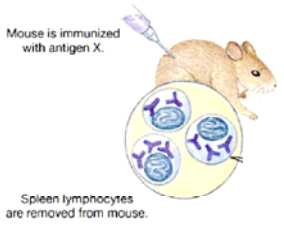
- Maintained biological activity 活性
- Sufficient purity and quantity 纯度 & 规模
- Good economy 经济性

设定目标：需要的蛋白量

终产品纯度要求
– 一般原则:

Extremely high 极高	High 高	Moderate 中等
• therapy 治疗用 • *in vivo* studies PK/PD 动物实验	• crystallization for x-ray studies 结晶 • N-terminal sequencing of an unknown protein N端测序 • most physical-chemical characterization methods 鉴定	• antigen for monoclonal antibody production 免疫





选择不同规模、不同功能的系统

BIOPROCESS SCALE

INCREASED THROUGHPUT
LAB SCALE

ÄKTAprocess™

ÄKTAxpress™

ÄKTApilot™

ÄKTAprime™ plus

ÄKTApurifier™

ÄKTAexplorer™

LAB SCALE
SIMPLE PURIFICATION

LOW THROUGHPUT
ADVANCED PURIFICATION

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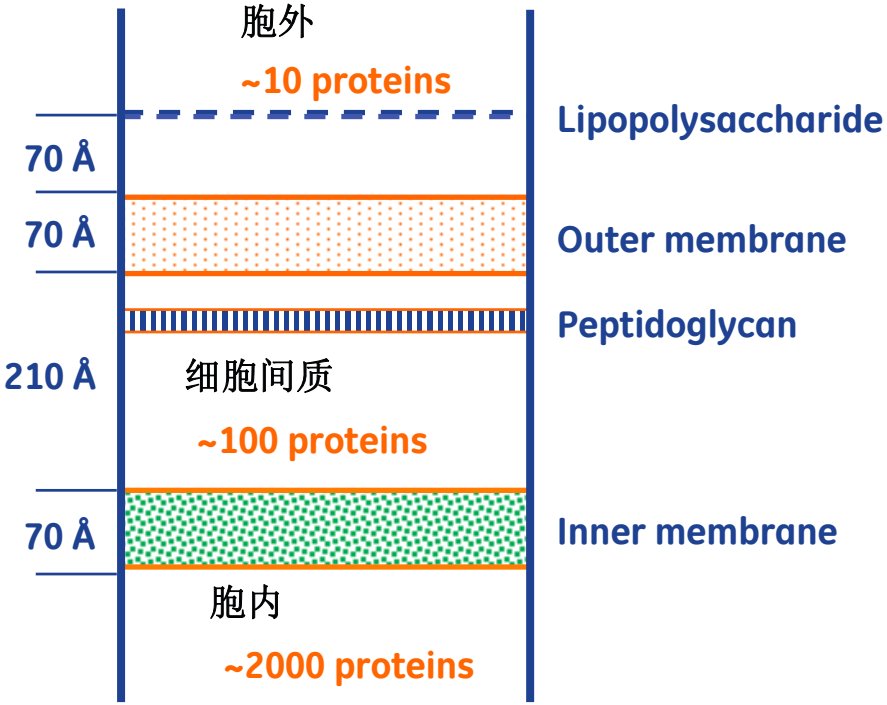
开始纯化之前

样品来源

- 原核 or 真核？
- 胞内 or 胞外？细胞破碎？
- 活性, 复性？

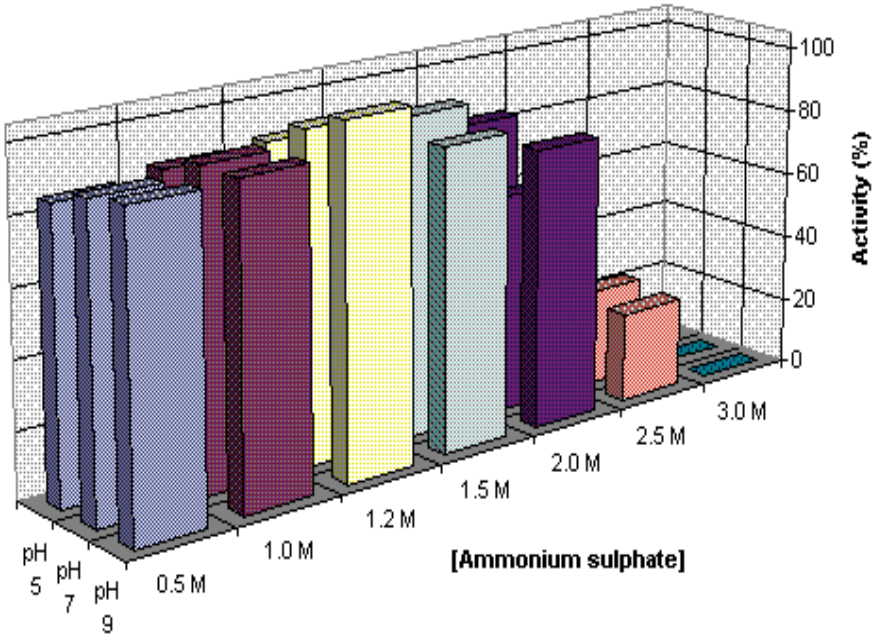
目标蛋白的性质

- 等电点
- 分子量
- 稳定性 (pH /salt, etc)



Target protein stability window
目标蛋白的稳定性

Determination of a suitable ammonium sulfate concentration and pH screening range for **HIC**

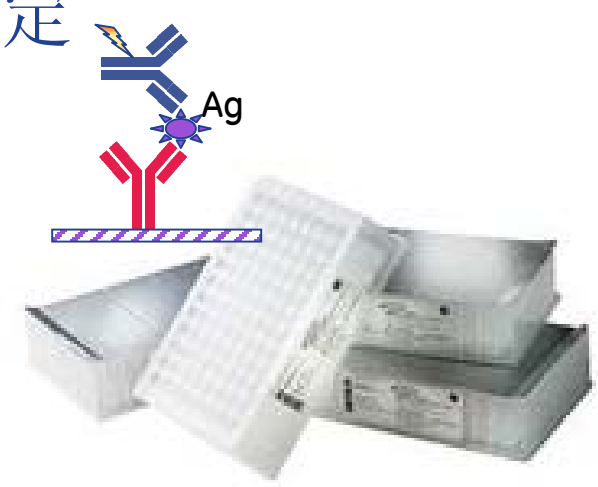


开始纯化之前

建立目标蛋白特异性检测方法

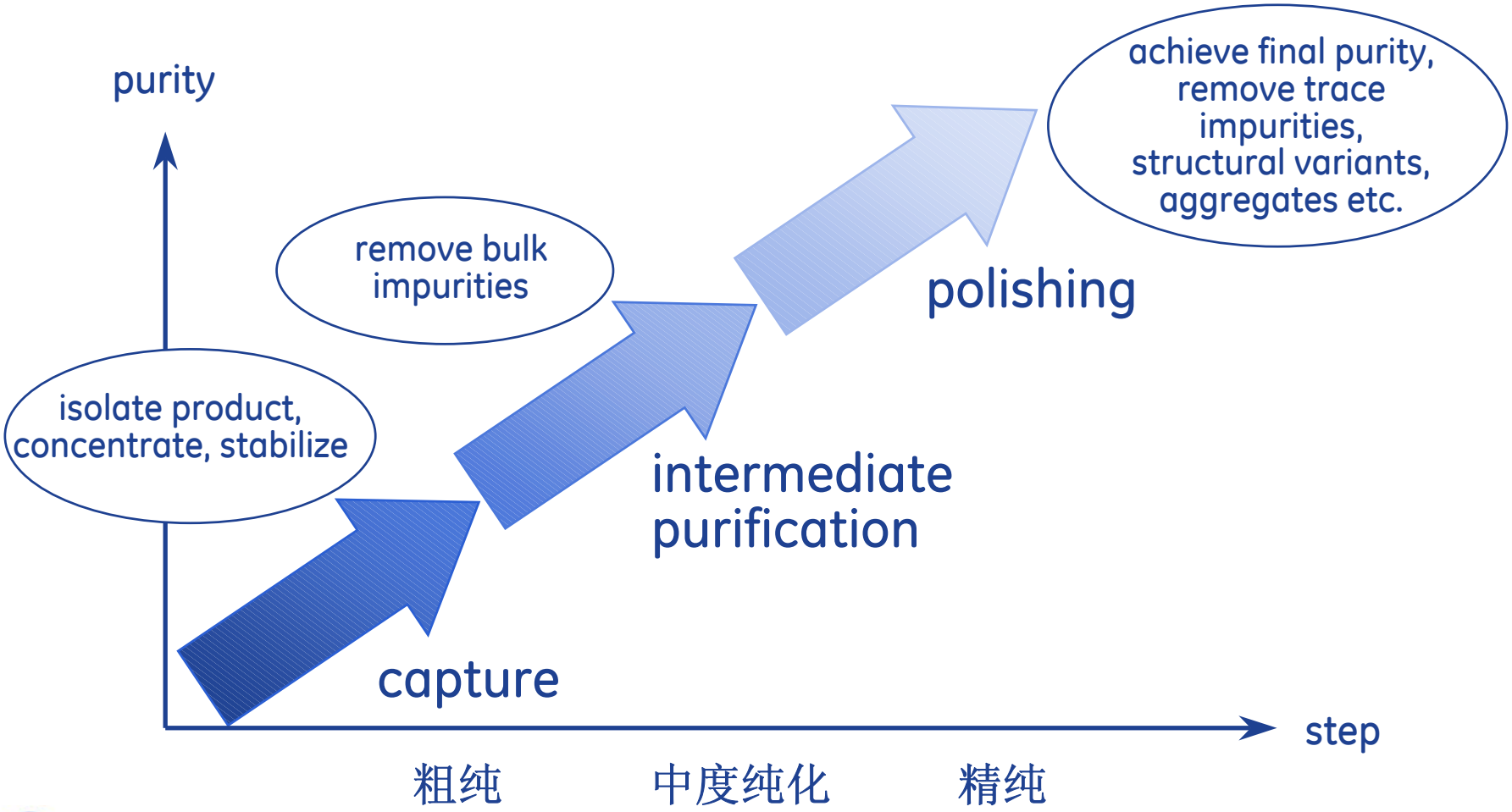
- Activity Assay (specific) 快速可靠的活性检测方法
  A rapid and reliable assay for the target protein
- Purity determination 纯度测定方法
  - e.g. SDS-PAGE电泳, HPLC高效液相层析
- Total protein determination 总蛋白测定
  - e.g. colorimetric method 比色法

主要杂质的性质及检测方法



Content

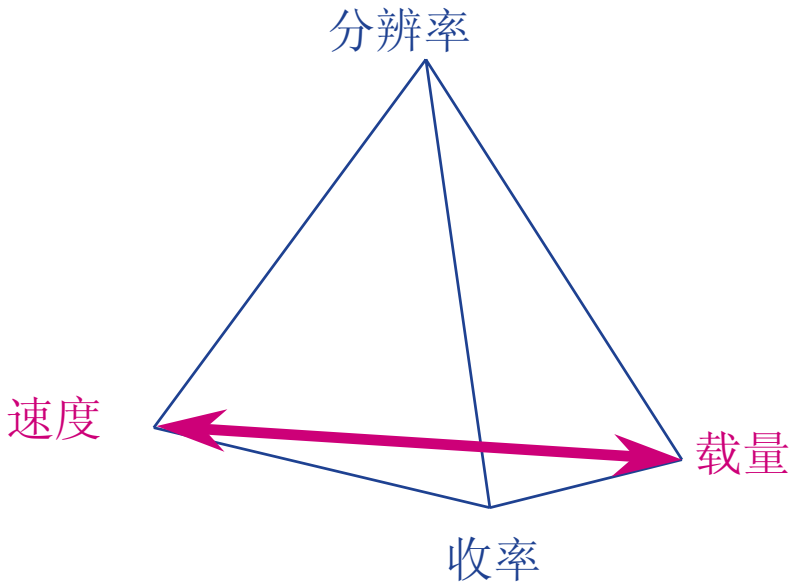
策略1：纯化三步曲(CIPP)
Three phase strategy



Capture 粗纯

Initial purification of the target molecule from crude or clarified source Material 样品为粗料液

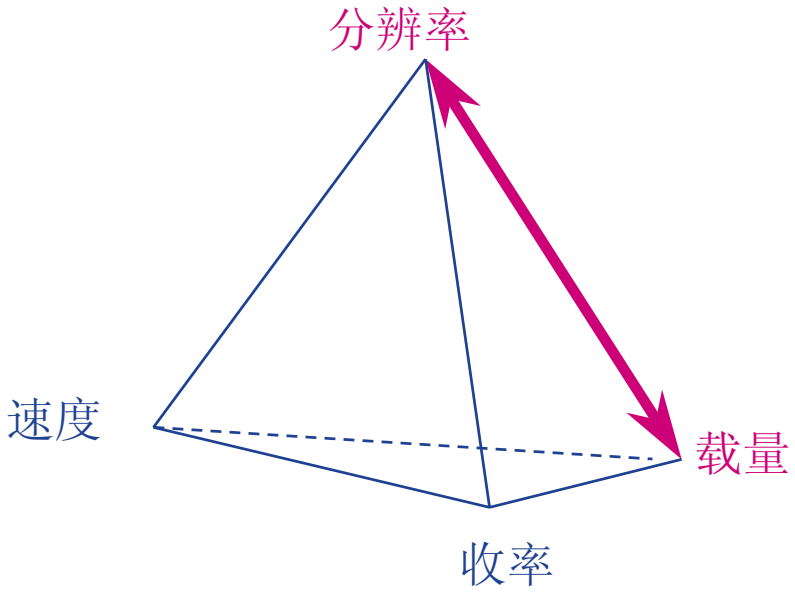
Concentration and stabilization
浓缩，稳定蛋白
- e.g. removal of proteases
  去除蛋白酶



Intermediate purification
中度纯化

Removal of bulk impurities

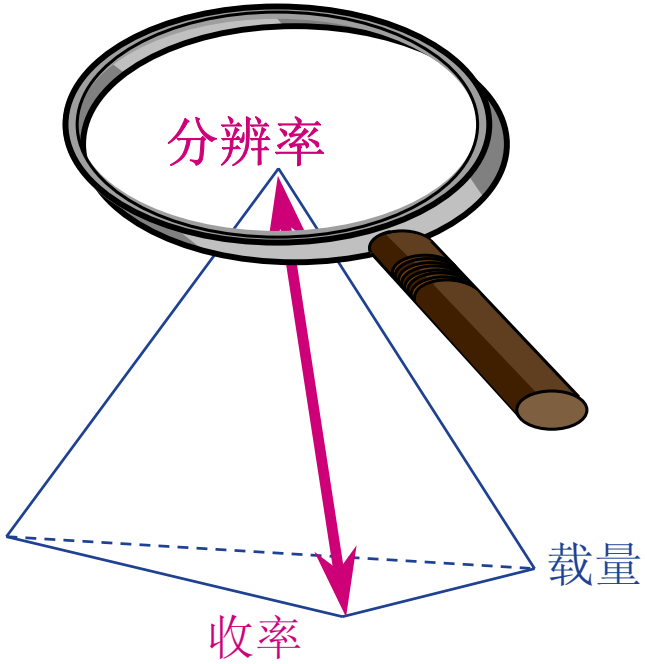
去除主要杂质



Polishing 精纯

Final removal of trace contaminants, e.g. structural variants of the target protein

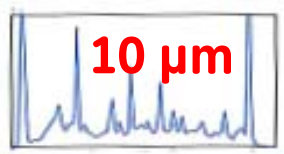
最终去除痕量杂质，如目标蛋白的结构变体等

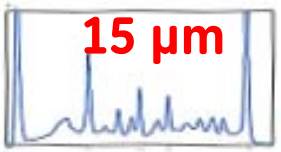


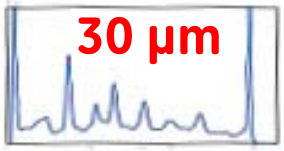
Three phase strategy 三步纯化策略
– Ranking of chromatography techniques

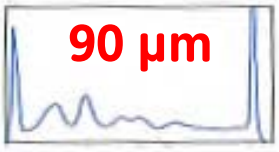
	Technique	Main features	Capture	Intermediate	Polish
离子交换	IEX	high resolution high capacity high speed	★★★	★★★	★★★
疏水	HIC	good resolution good capacity high speed	★★	★★★	★
亲和	AC	high resolution high capacity high speed	★★★	★★★	★★
分子筛	GF	high resolution using Superdex		★	★★★
反相	RPC	high resolution		★	★★★
			粗纯	中度纯化	精纯

Impact of bead sizes 粒径的影响

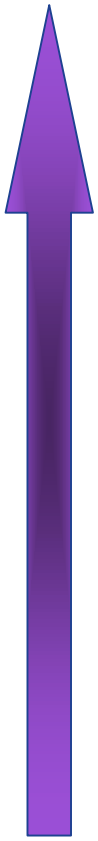








MiniBeads™	3 µm	micro-purification
MonoBeads™	10 µm	polishing
Superdex™	13 µm	polishing
SOURCE™ 15	15 µm	polishing
SOURCE 30	30 µm	intermediate
Sepharose™ HP	34 µm	intermediate
Superdex pg	34 µm	polishing
Sepharose FF	90 µm	capture
Capto™	90 µm	capture



分辨率
反压

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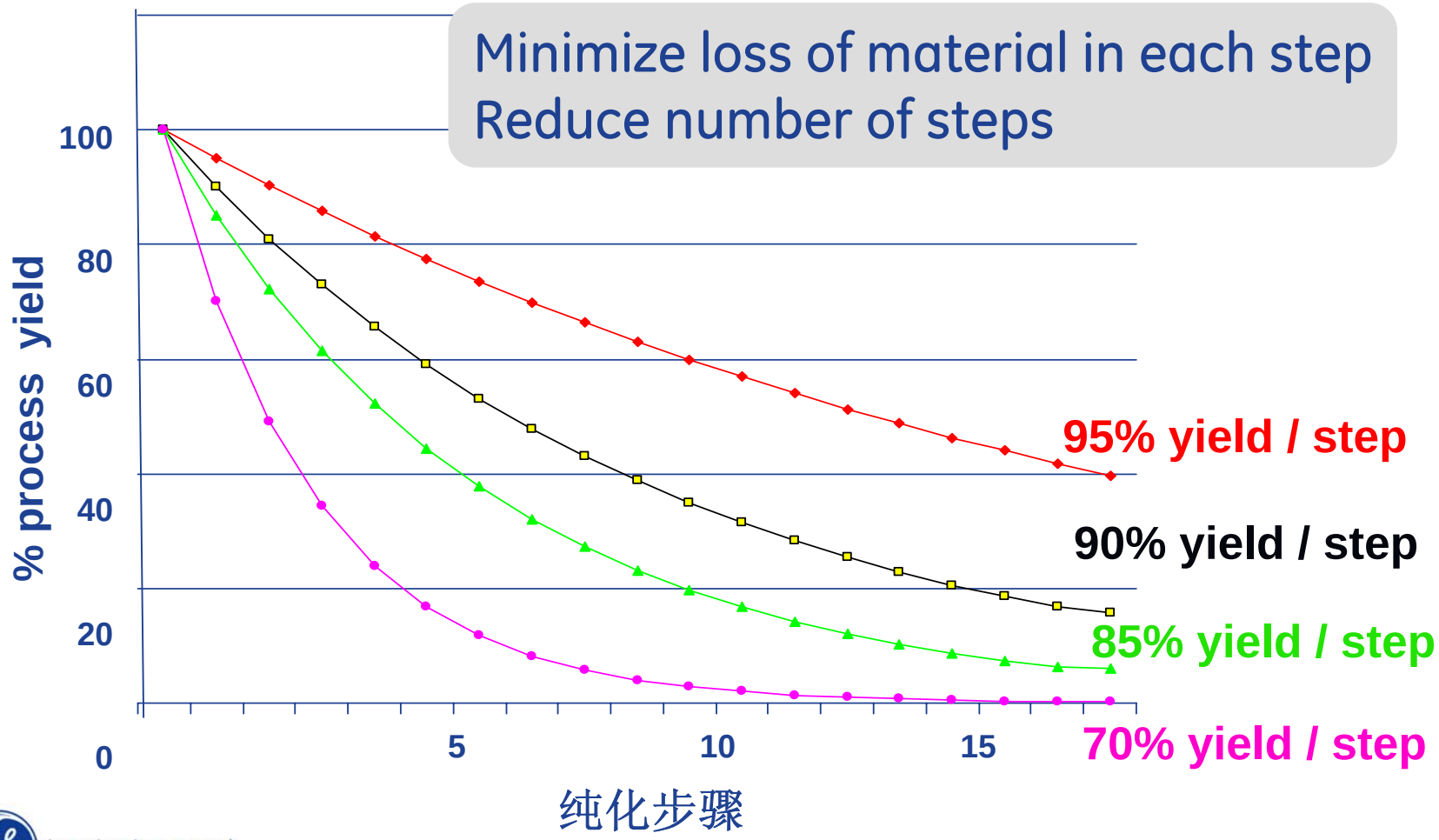
策略2：步骤越少,收率越高

Yields from multi-step protein purifications



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纯化表

一种膜结合蛋白, 白细胞三烯 C4 合成酶, 18 kDa protein

宿主: *Pichia pastoris 毕赤酵母*

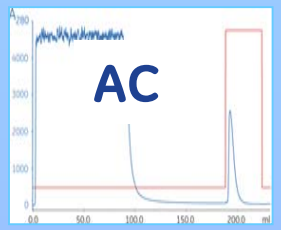
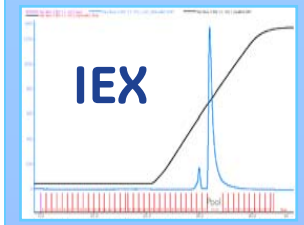
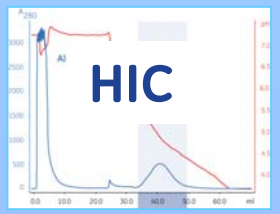
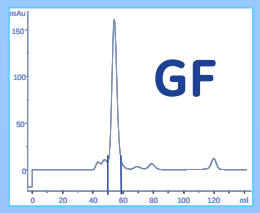
纯化步骤	体积 ml	蛋白浓度 mg/ml	总蛋白量 mg	总活性 µmol	比活 µmol/mg/min	纯化倍数 fold	活性收率 %	
匀浆	220	1.7	374	108	0.29	1	100	
溶解	226	1.74	393	314	0.8	2.76	291	100
亲和 1	30	0.13	3.9	76	19.5	67	70.4	24.2
亲和 2	19	0.14	2.9	29	9.6	33	26.8	9.2
脱盐/浓缩	1	1.14	1.1	35	28	97	32.6	11.1

Wetterholm *et. al.*, Prot Expr. Purif. 60 (2008) 1-6

策略3：交替运用互补技术,合理衔接

- 将分离机理互补的技术进行组合, 交替运用不同的层析方法 (e.g. IEX, HIC and SEC)
- 减少不同层析技术间的样品处理 (e.g. 浓缩, 置换缓冲液)
- 尽量简单

综合考虑起始和结束条件进行衔接

技术				
结合条件	特定条件	低盐	高盐	不结合
洗脱条件	特定洗脱条件	高盐	低盐	常液洗脱
浓度	增加	增加	增加	降低

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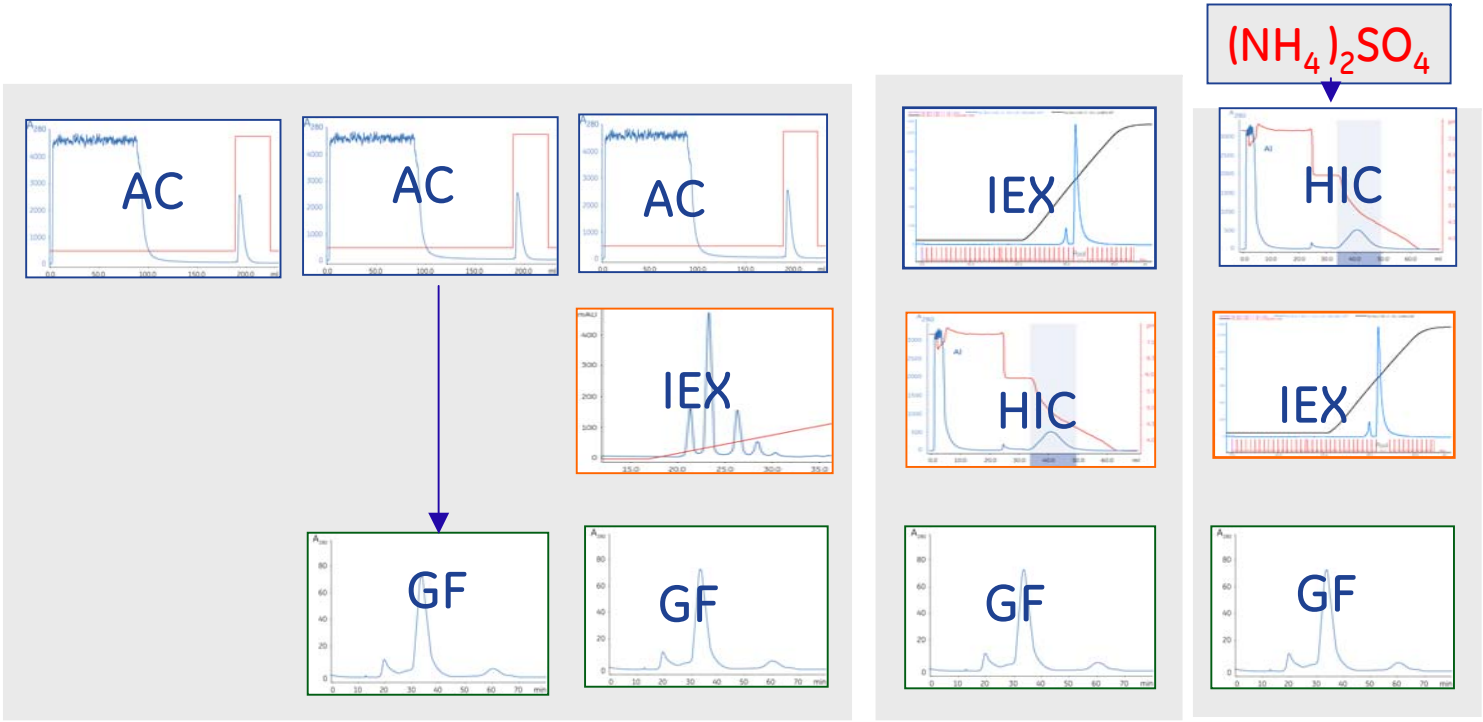
设计合理的纯化路线



Combine techniques 使用不同层析技术

complementary selectivities 选择性互补

minimized sample handling 尽量避免样品处理步骤

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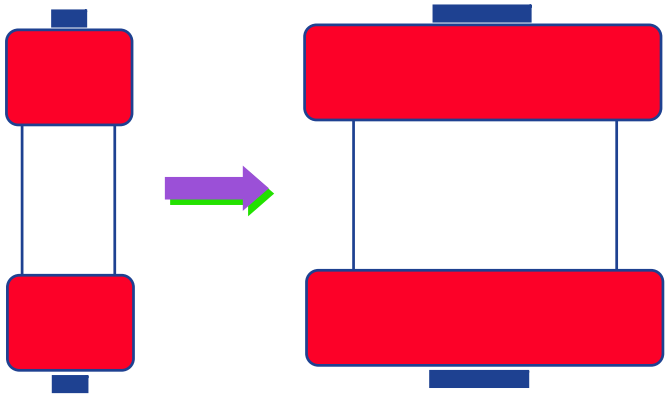
策略4：线性放大

固定以下条件：

1. 相同填料
2. 相同缓冲液
3. **相同线性流速/相同柱高**
4. 相同样品浓度和缓冲条件
5. 相同样品体积和柱床体积比例
6. 相同梯度体积和柱床体积比例



放大：

1. 柱直径
2. 体积流速
3. 上样体积

线性流速: $cm/h = \frac{ml/min \times 60}{cm^2}$

停留时间: $min = \frac{cm \times 60}{cm/hr}$

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Scale-up: 从实验室到工业生产规模

ÄKTAexplorer
快速全自动工艺开拓系统

ÄKTApilot
卫生级中试层析系统

ÄKTAprocess
大规模生产层析系统

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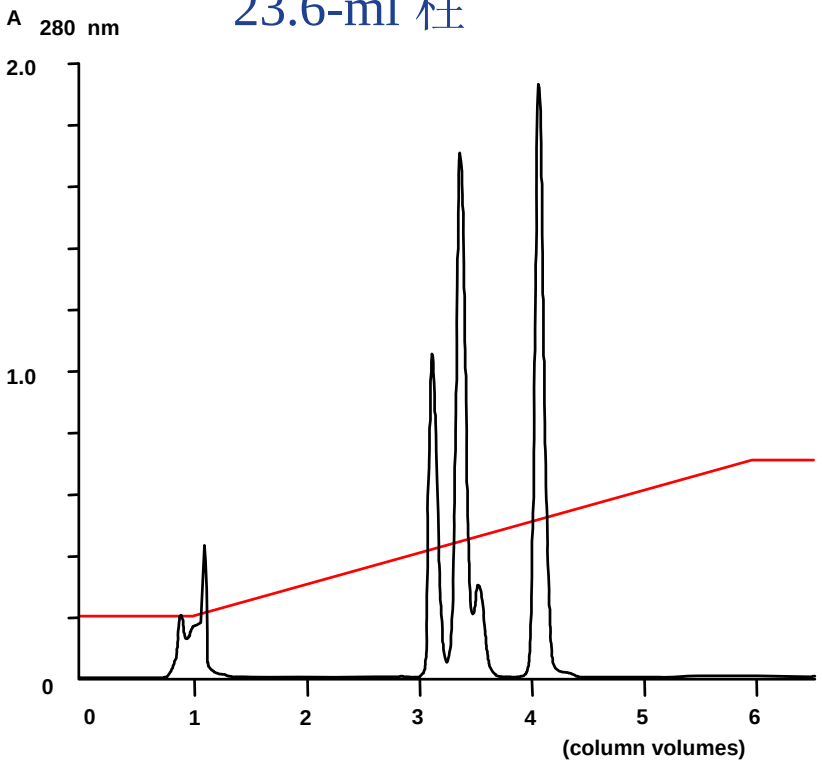
Scale-up: 从实验室到工业生产规模

ÄKTA™ explorer10

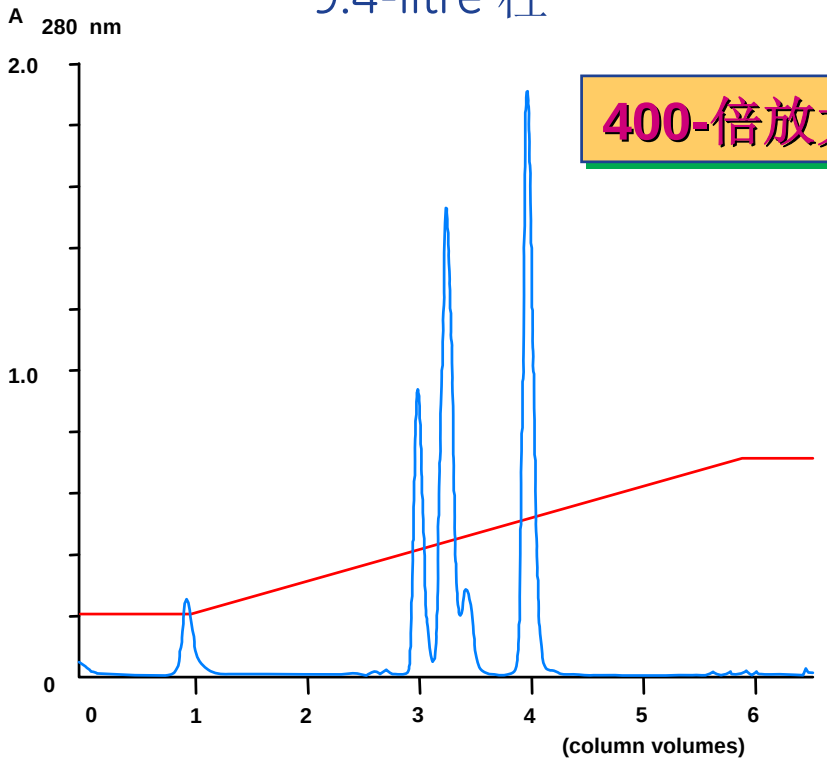
23.6-ml 柱



10 mm 直径柱

ÄKTA™ process

9.4-litre 柱



400-倍放大

FineLINE 200L

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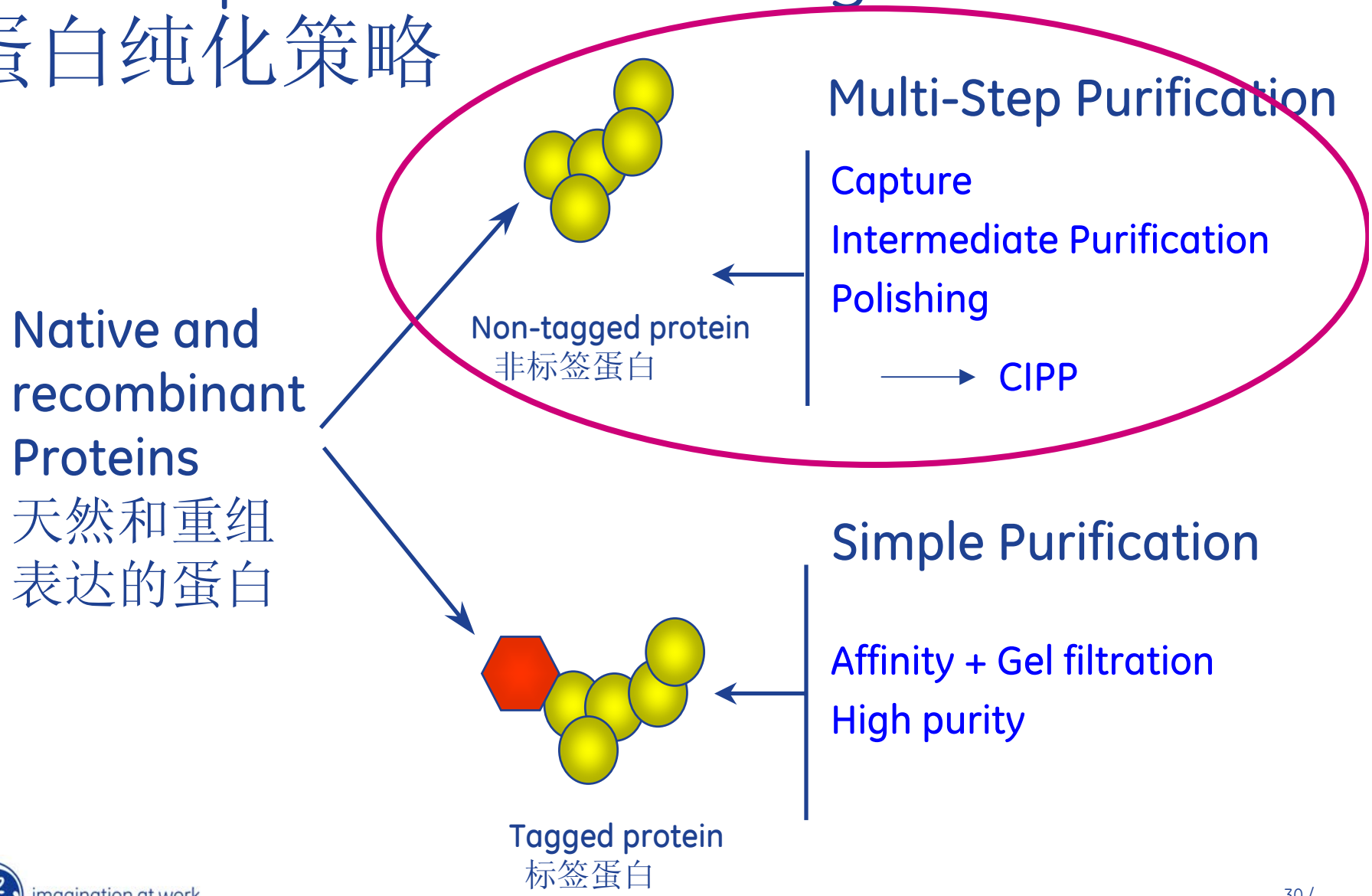
同一ÄKTA平台上的直接线性放大

Content

Protein purification strategies
蛋白纯化策略



DAOCS - 纯化策略
去乙酰氧化头孢菌素 C 合成酶

- 精细纯化
- 中度纯化
  - 快速探索填料 (scouting)
  - 快速探索梯度 (scouting)
- 粗纯
  - 快速探索填料 (scouting)
  - 快速探索梯度 (scouting)
- 准备样品
- DAOCS·性质
- 纯化目标

得到 5-10 mg DAOCS，纯度足够进行结晶和X-晶体衍射分析
一个工作日内完成整个纯化流程

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DAOCS – 目标蛋白的性质

参数	数值	对纯化设计的影响
等电点	4.8	在粗纯时使用中性 pH 进行阴离子交换层析
分子量	34 500	用 Superdex™ 75 prep grade 凝胶过滤技术进行精细纯化 (3-70kD)
盐稳定性	>2 M $(NH_4)_2SO_4$	可以使用疏水层析
一般稳定性	容易氧化	在缓冲液中加 DTT 工艺设计重点缩短整体纯化时间

DAOCS – 注意事项

在所有缓冲液中加 DTT 使所有半胱氨酸残基保持还原状态, 防止氧化

加入蛋白酶抑制剂以防止蛋白酶解

用 SDS-PAGE 检查每一个组份中 DAOCS

终产品通过酶活测定来确认生物活性

样品预处理

细菌菌体重悬于裂解缓冲液中

↓

超声破碎菌体

↓

沉淀DNA (optional)

↓

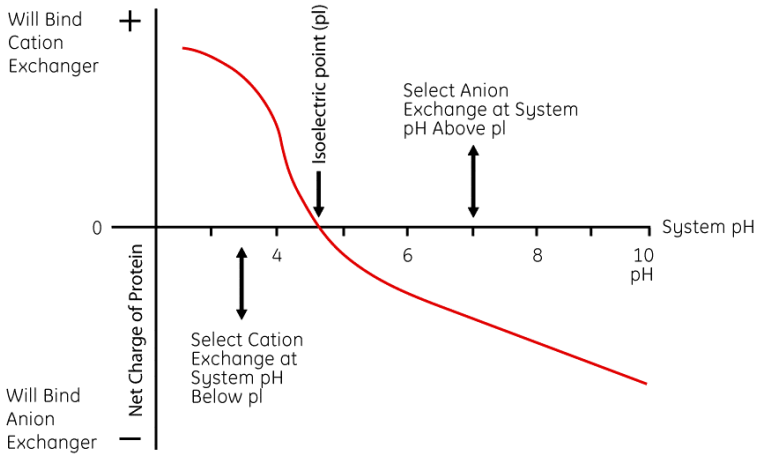
*20,000xg*离心澄清

来源:从 *S. Clavuligerus* 克隆得到的DAOCS 基因 和在大肠杆菌的胞浆中扩增

选择粗纯的层析技术

pI = 4.8, 阴离子交换:

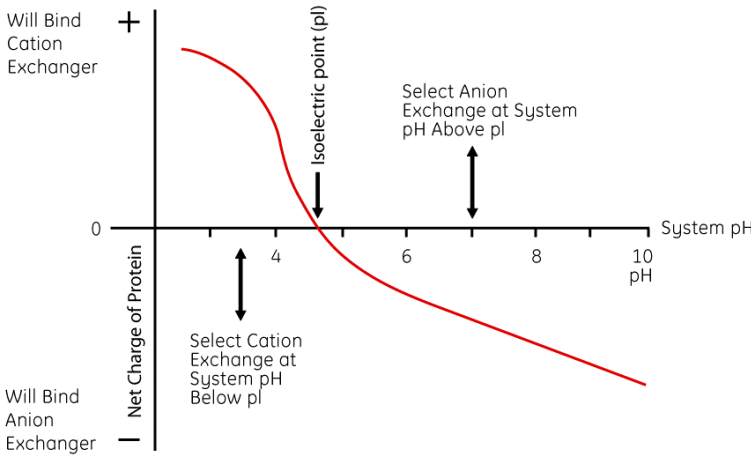
- 快速, 浓缩
- 样品处理简单

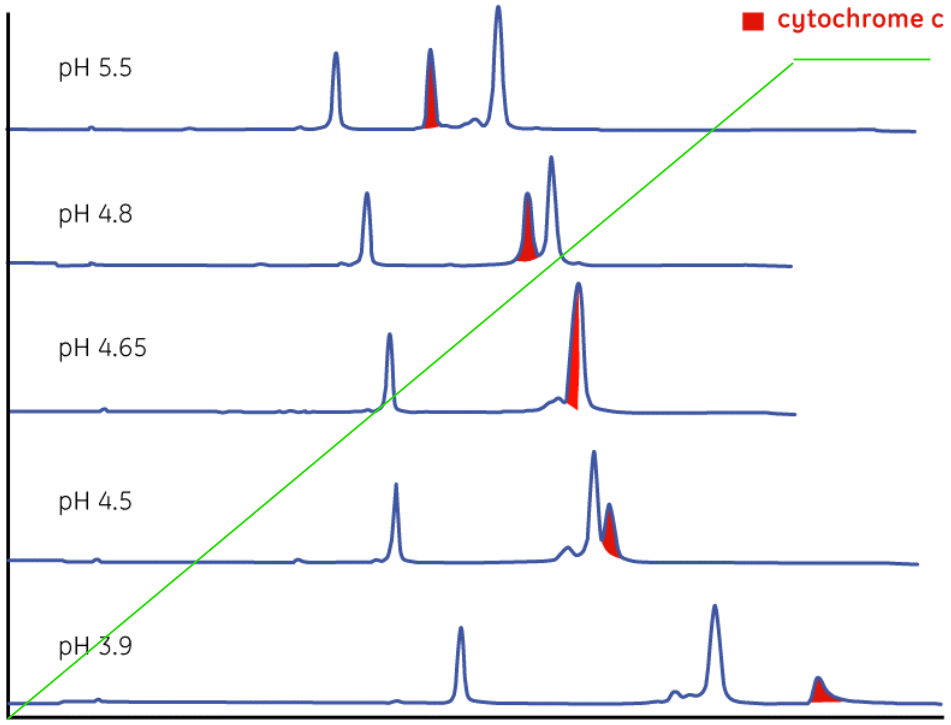


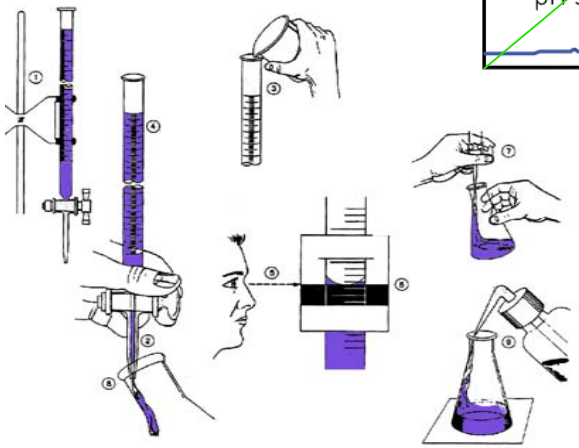
分离机理: 带电性质差异

考虑到DACOS蛋白的酸性 pI 和稳定性, 缓冲液选择中性 pH

pH的选择 (关键参数)



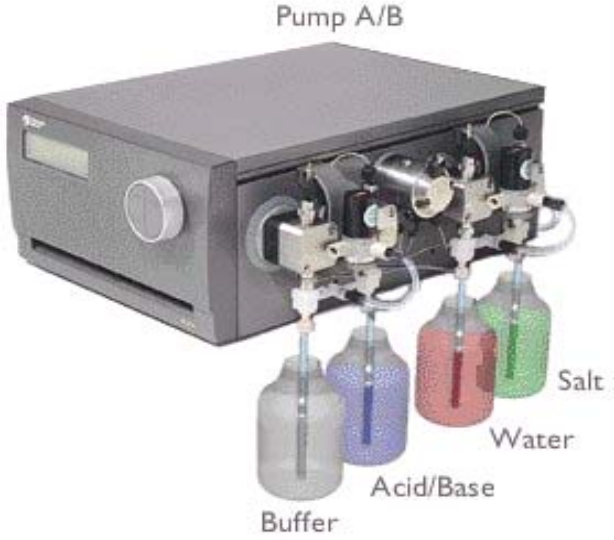


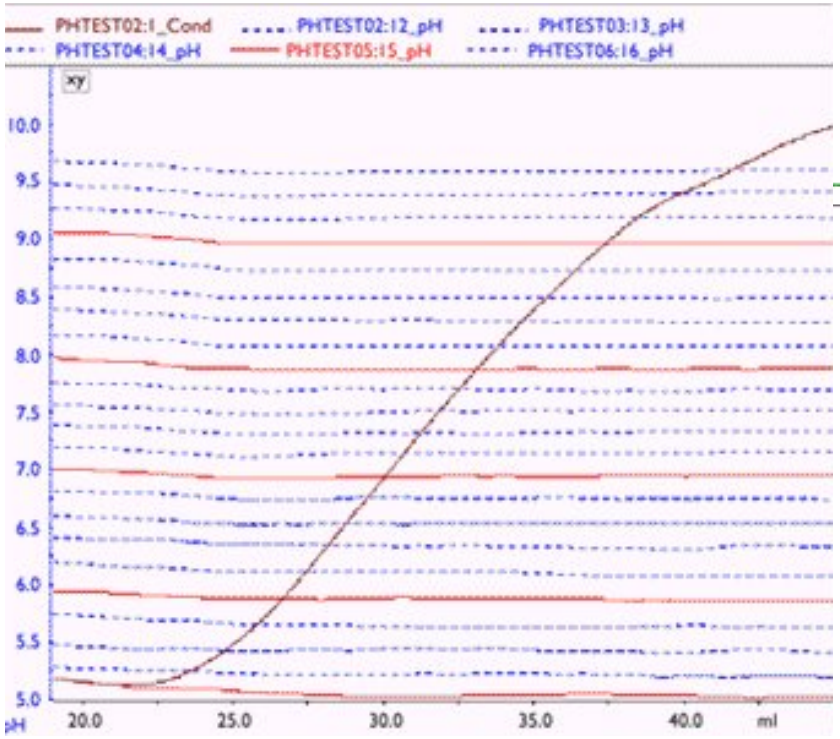


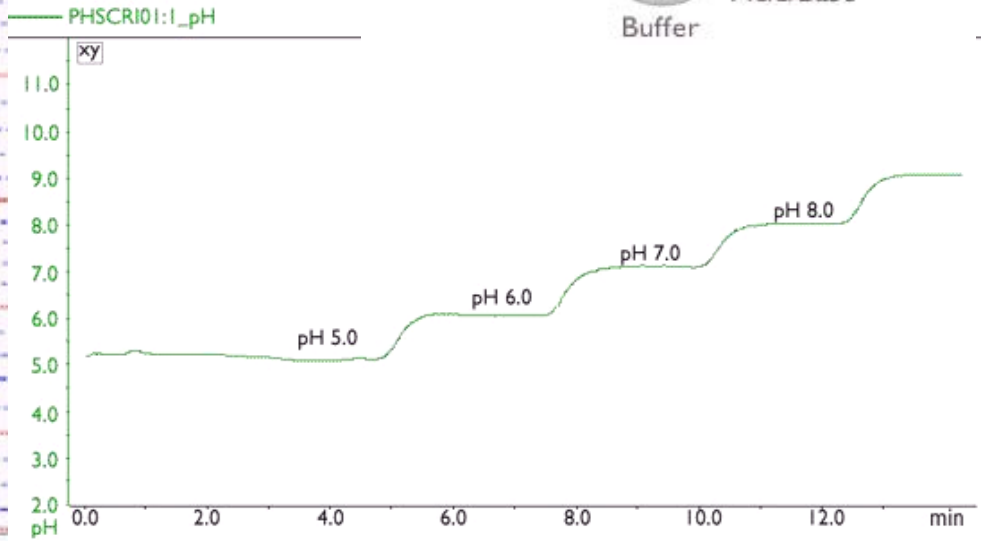
imagination at work

ÄKTA 自动缓冲液制备 BufferPrep

Buffer is titrated on pump A
Pump B controls salt conc.





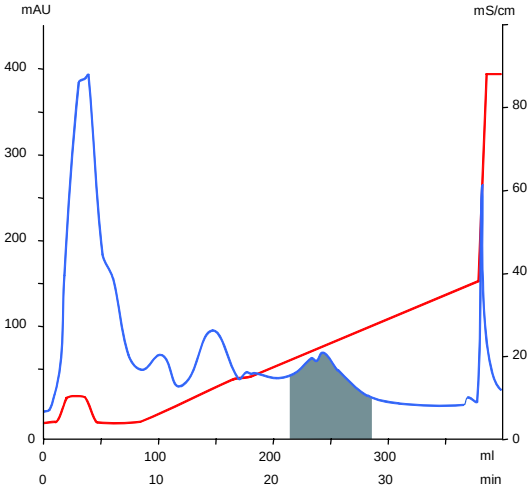


imagination at work

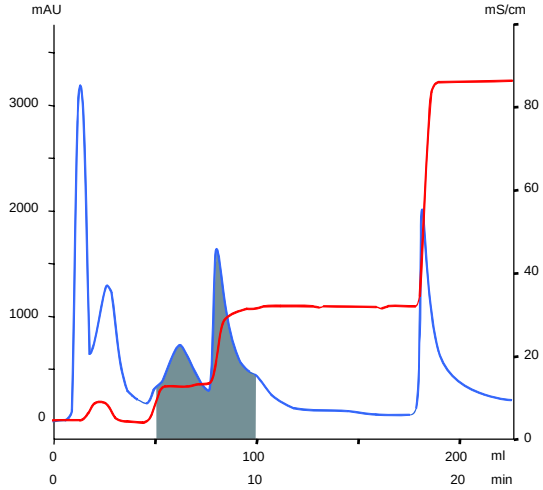
粗纯 - 在 ÄKTA™上优化梯度

阴离子交换层析柱: HiPrep™ 16/10 **Q XL**

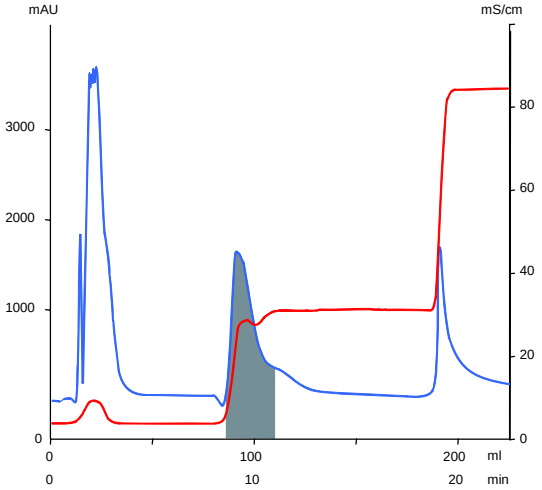
线性梯度



阶段梯度



优化梯度



选择中度纯化技术

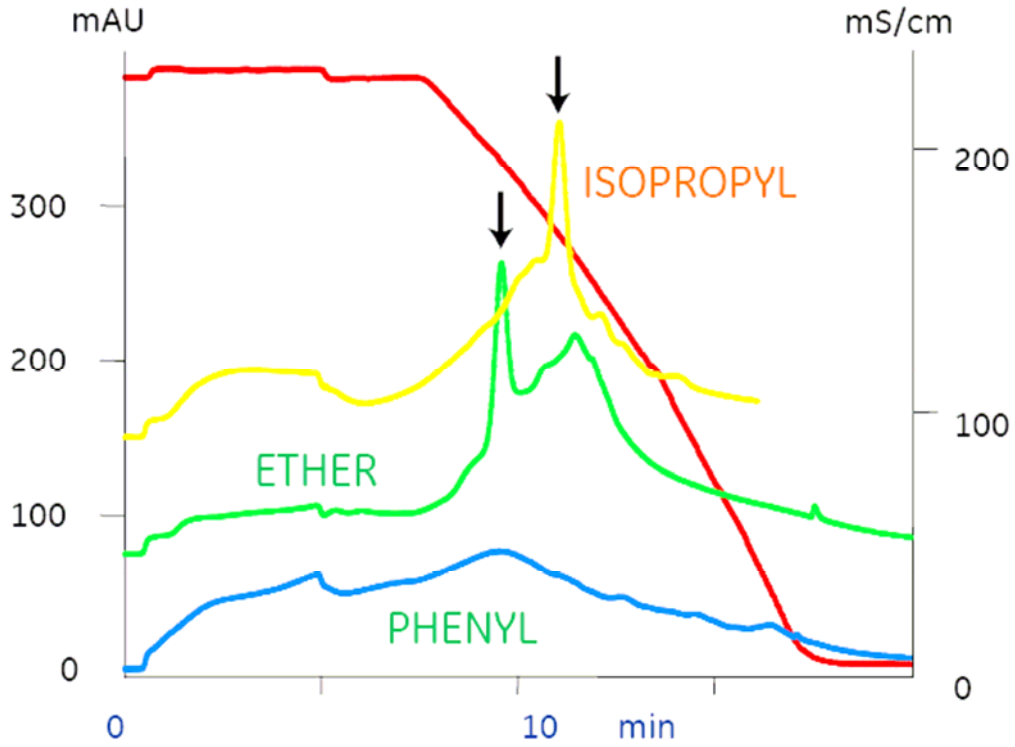
疏水层析HIC:

- 可以跟 IEX 互补衔接, 减少样品处理步骤 (只需加盐)
- DAOCS 在高盐下能保持稳定

分离机理: 疏水性差异

蛋白质疏水性质很难预测: 筛选适合填料

中度纯化 – 疏水填料筛选



Columns:	RESOURCE™ ISO 异丙基 RESOURCE ETH 醚基 RESOURCE PHE 苯基
Sample vol:	10 ml
Buffer A:	2 M ammonium sulfate, 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH7.5
Buffer B:	buffer A without ammonium sulfate
Flow rate:	3 ml/min
System:	ÄKTAFPLC™

ÄKTA crystal系统 (自动多维纯化)

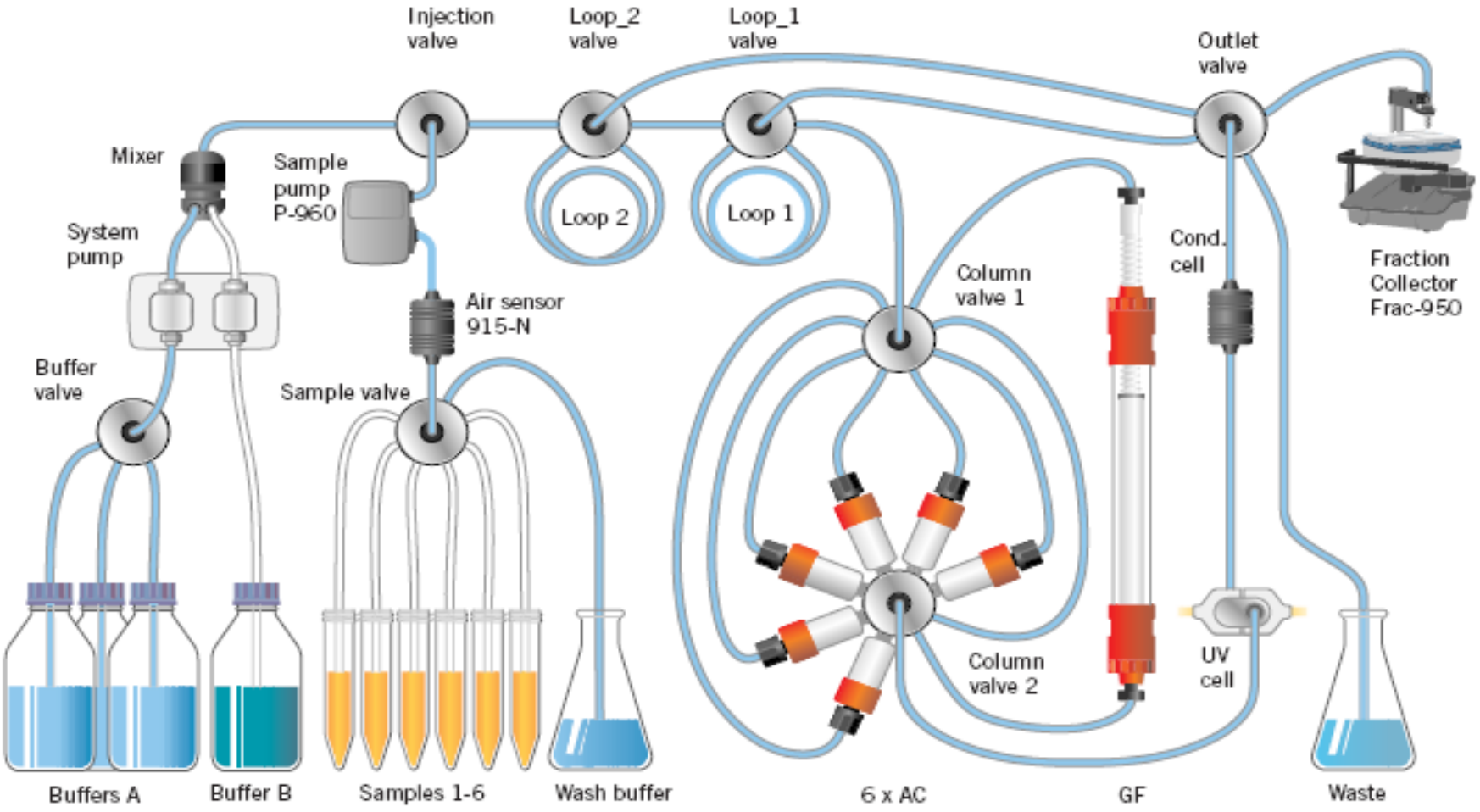


Fig 1-1. Schematic drawing of ÄKTAexplorer 100 together with ÄKTA 3D plus Kit (setup for protocol C: AC-GF).

选择精细纯化层析技术

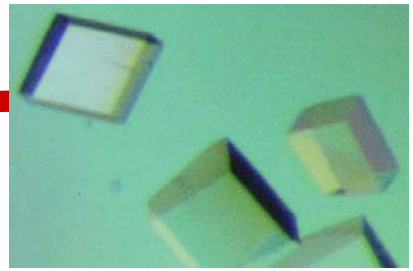
分子筛 SEC:

- 简单
- 有力的补充IEX和HIC

分离机理: 分子量差异

最适合分离二聚体, 寡聚体和聚合物

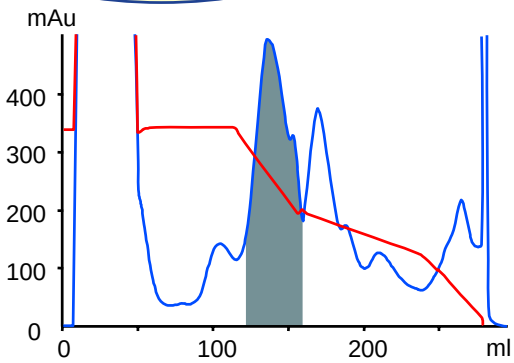
DAOCS 纯化 - 结果



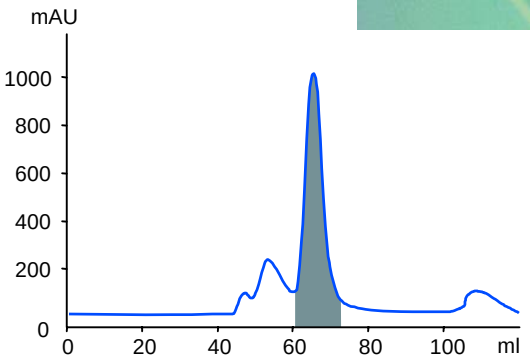
精细纯化

中度纯化

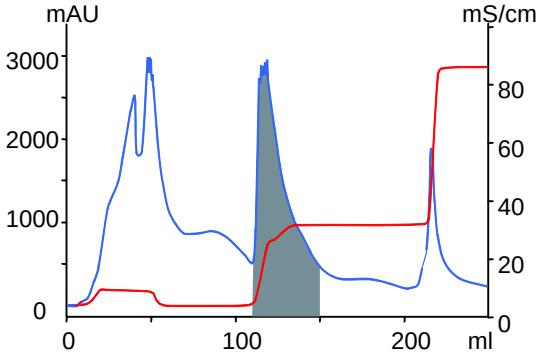
粗提



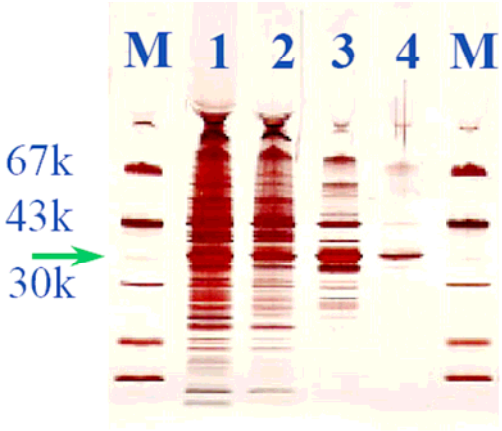
SOURCE™ 15 ISO, 内径 16 mm, 长度 50 mm



HiLoad™ 16/60 Superdex™ 75 prep grade



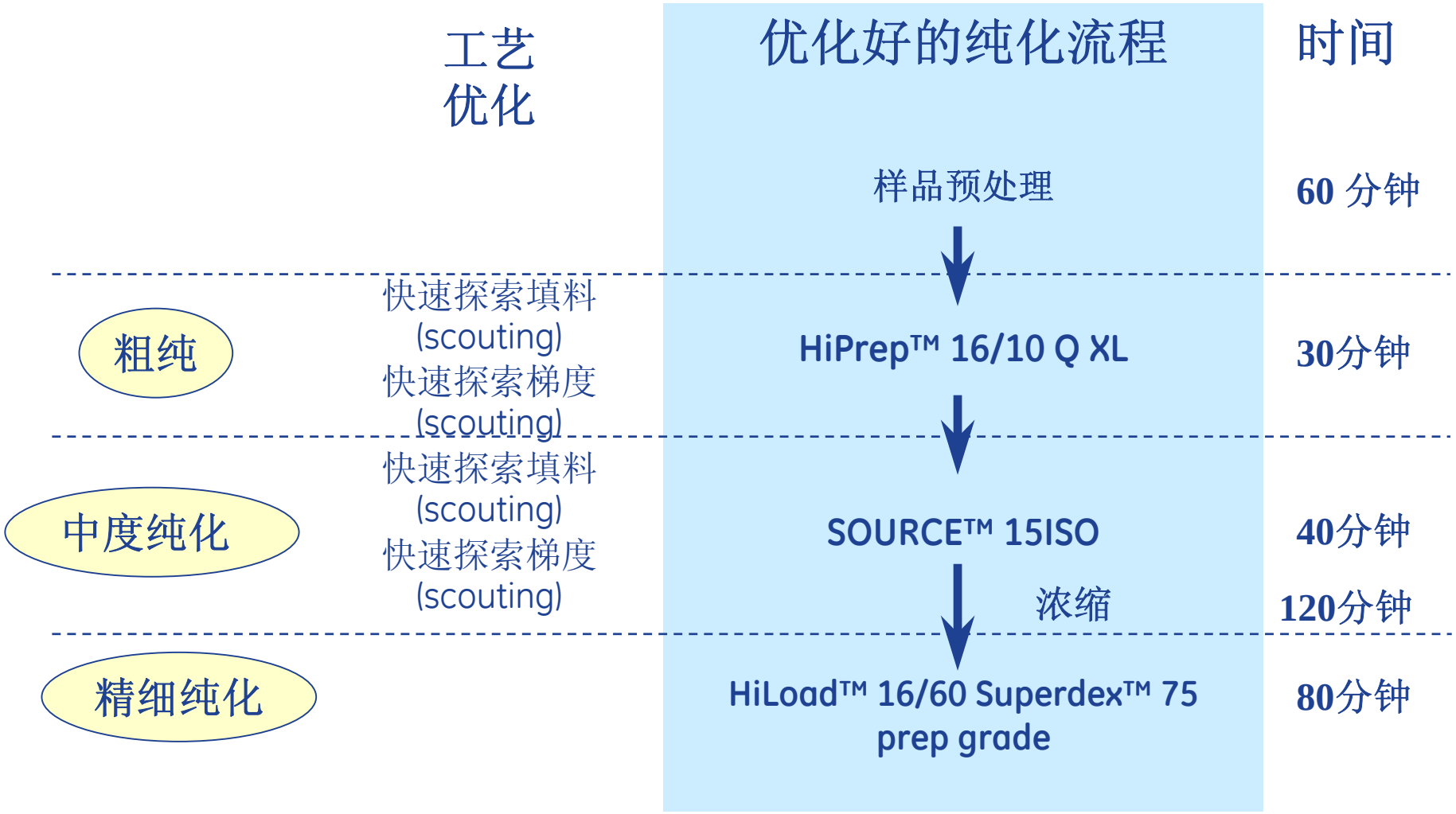
HiPrep™ 16/10 Q XL



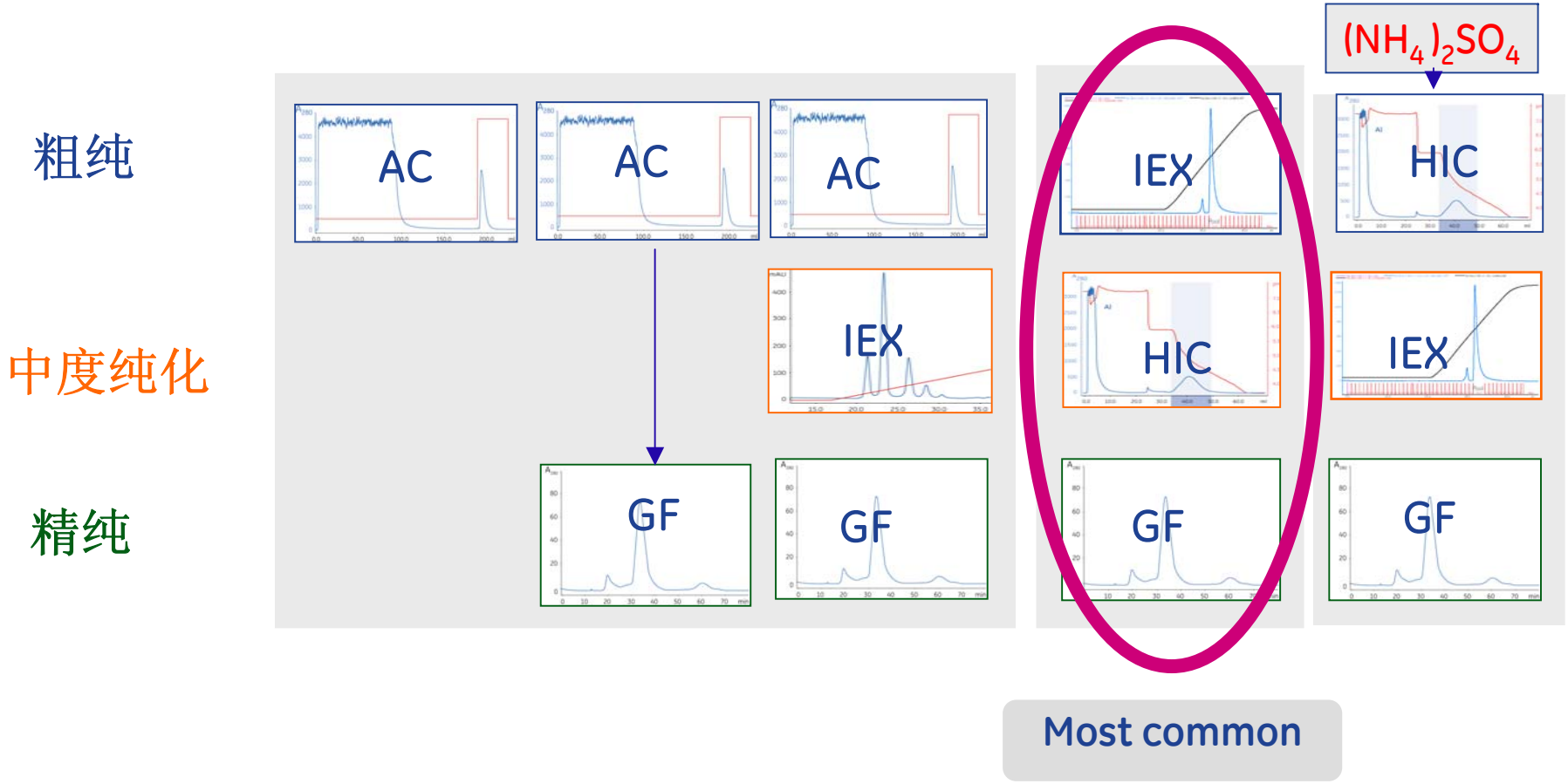
M - LMW 低分子量marker
1 - 原始样品
2 - Q Sepharose™ XL 洗脱峰
3 - SOURCE™ 15ISO 洗脱峰
4 - Superdex™ 75 pg 洗脱峰

imagination at work

DAOCS 纯化 - 总结


工艺
优化
优化好的纯化流程
时间
样品预处理
60 分钟
粗纯
快速探索填料
(scouting)
快速探索梯度
(scouting)
HiPrep™ 16/10 Q XL
30分钟
中度纯化
快速探索填料
(scouting)
快速探索梯度
(scouting)
SOURCE™ 15ISO
40分钟
浓缩
120分钟
精细纯化
HiLoad™ 16/60 Superdex™ 75
prep grade
80分钟

层析技术的衔接



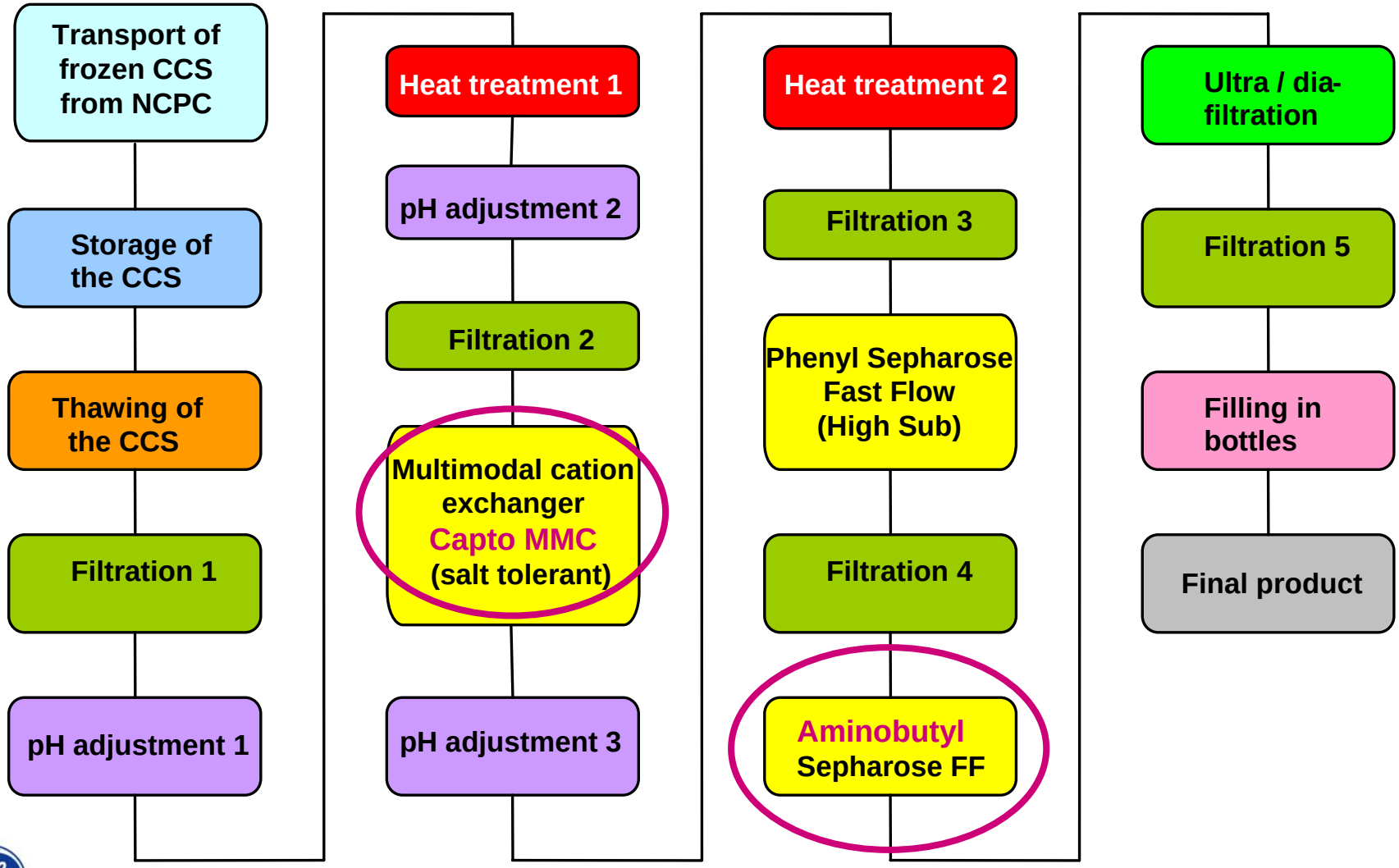
Generic purification strategy !

通用的工艺路线！

从毕赤酵母纯化重组的α-甘露糖苷酶

Technique		Purification factor	Comment
			Y.-F. Liao et al. (1996) J. Biol. Chem. 271, 28348-28358
UF	Ultrafiltration		
AIEX	Q Sepharose™ Fast Flow	63	• Capture with step gradient; 730 mg of total protein applied
HIC	Phenyl Sepharose High Performance	622	
GF	Superdex™ 200 prep grade	719	• 83 mg homogenous protein obtained

重组人白蛋白的下游纯化工艺

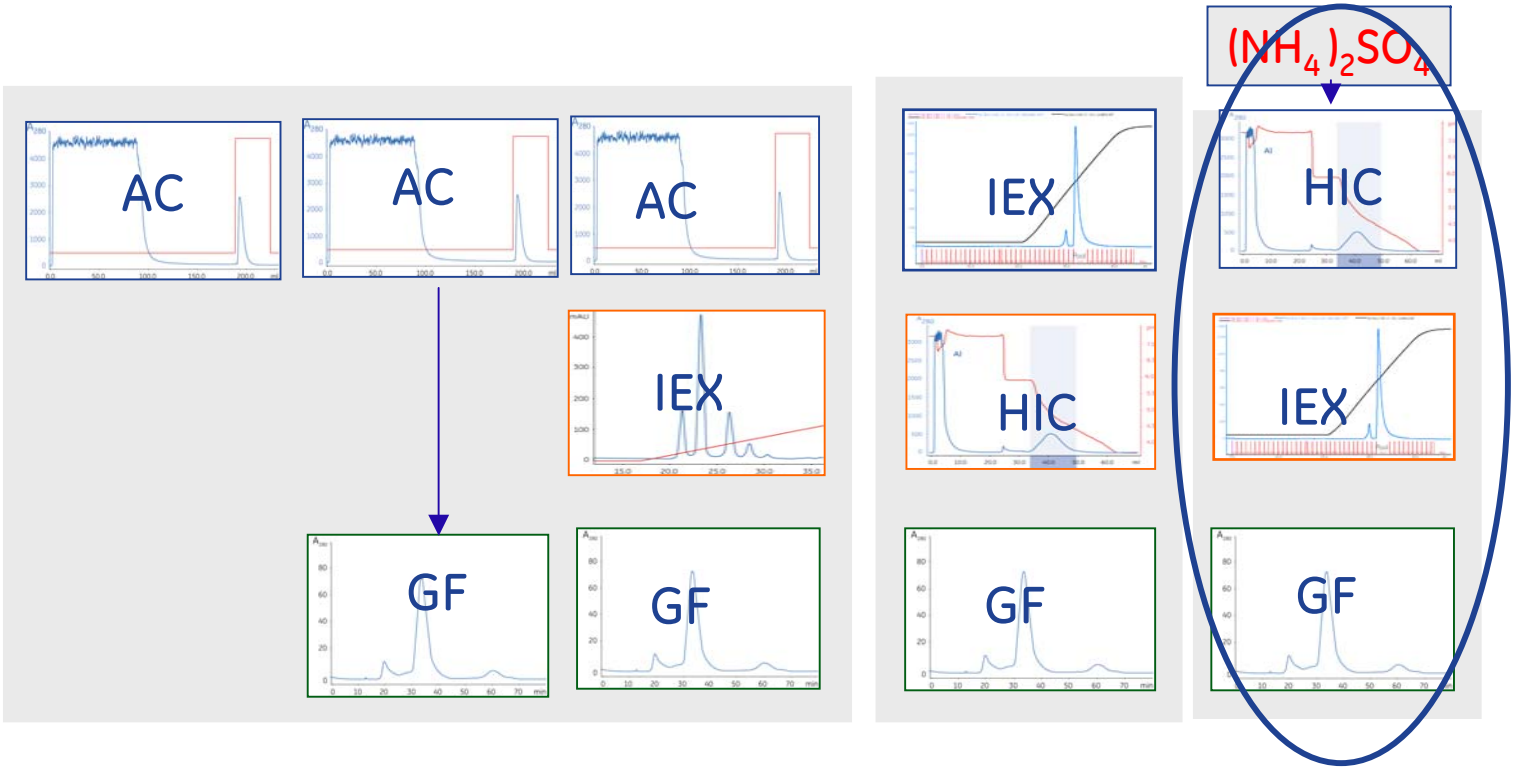


层析技术的衔接

捕获

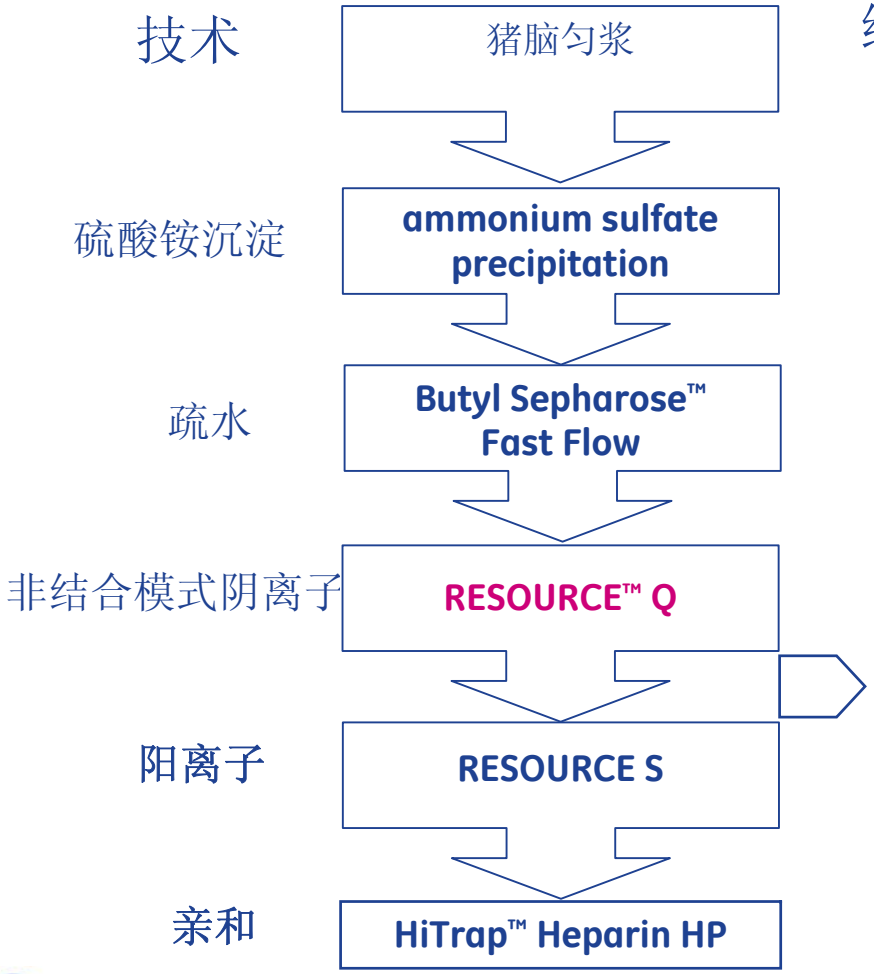
中度纯化

精细纯化



Hydrophobic & membrane proteins

G protein receptor kinase purification
G蛋白受体激酶的纯化



Comment

A. Tobin et al. (1996)
J. Biol. Chem. 271, 3907-3916

- 缓冲液中加入蛋白酶抑制剂
- 4度下纯化
- 杂质结合
- 洗脱条件作为下一步骤结合条件
- 得到10 mg 均一蛋白

imagination at work

Purification of rhGM-CSF (MW14.6k, pI 5.4)
重组人粒细胞-巨噬细胞集落刺激因子

E. coli cells

Inclusion bodies 包含体

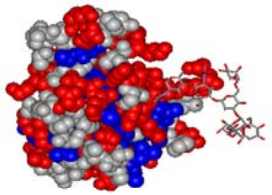
Washed pellet 沉淀清洗 ← 溶解 (6 M GuHCl)

Renatured rhGM-CSF 复性 ← 稀释复性

HIC — Phenyl Sepharose™ 6 Fast Flow (high sub)

AIEX — Q Sepharose Fast Flow

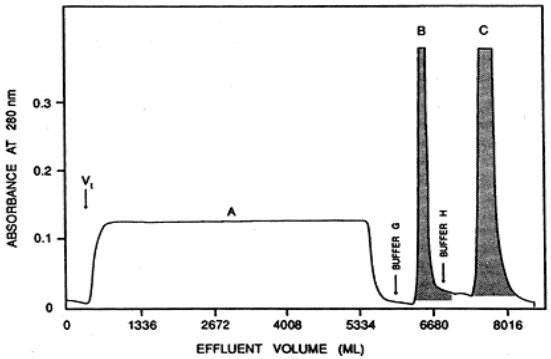
GF — Superdex™ 75 prep grade



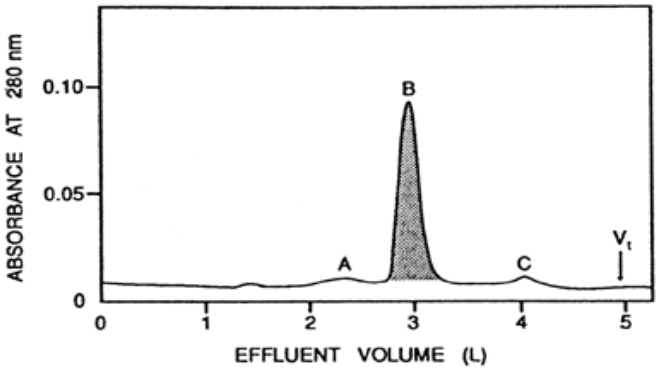
imagination at work

rhGM-CSF的纯化

Step 1. Phenyl Sepharose™ 6 Fast Flow (high sub)



Step 3. Superdex™ 75 prep grade

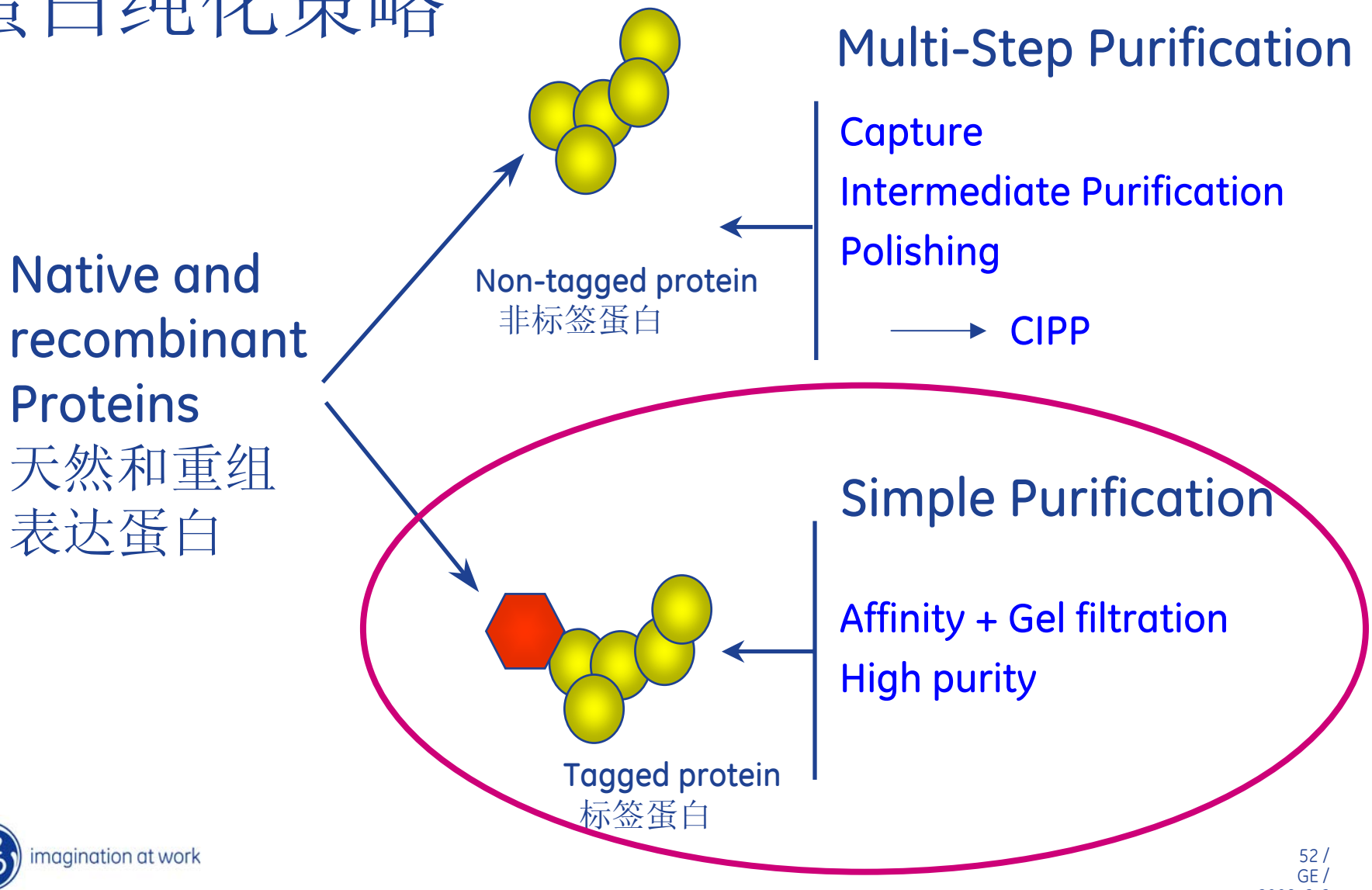


Step	Total vol ml	Total protein mg	Total Endotoxin EU	Endotoxin Clearance	Total DNA ng	DNA Clearance
Start	4344	490	1828824	-	781920	-
1. HIC	880	220	213840	8.6	405	1931
2. AIEX	620	88	155620	1.4	87	4.66
3. GF	1045	62	9405	16.5	84	1.04

imagination at wc

Specific activity of final product : ca. 3.3 x 10e7 units/mg,

Protein purification strategies
蛋白纯化策略



带标签的融合表达的重组蛋白

优势：

简单的亲和纯化

检测方便

可以进行柱上复性

产率提高 (expression/stability)

溶解度提高/folding

劣势：

可能需要去除标签

标签可能干扰结构或功能

不同的标签

Histidine

+ Small
+ Cleavage often not needed
+ Many references
+ Cheap
-Inclusion bodies
-Non-specific purification

GST

+ Increases solubility & yield
+ Specific purification
-Large
-Cleavage often necessary
-Binding capacity

MBP

+ Increases solubility & yield
+ Specific purification
-Large
-Cleavage often necessary

***Strep* tag II**

+ Small
+ Cleavage often not needed
+ Very specific purification
-Expensive
-Not many references yet

标签蛋白的纯化趋势

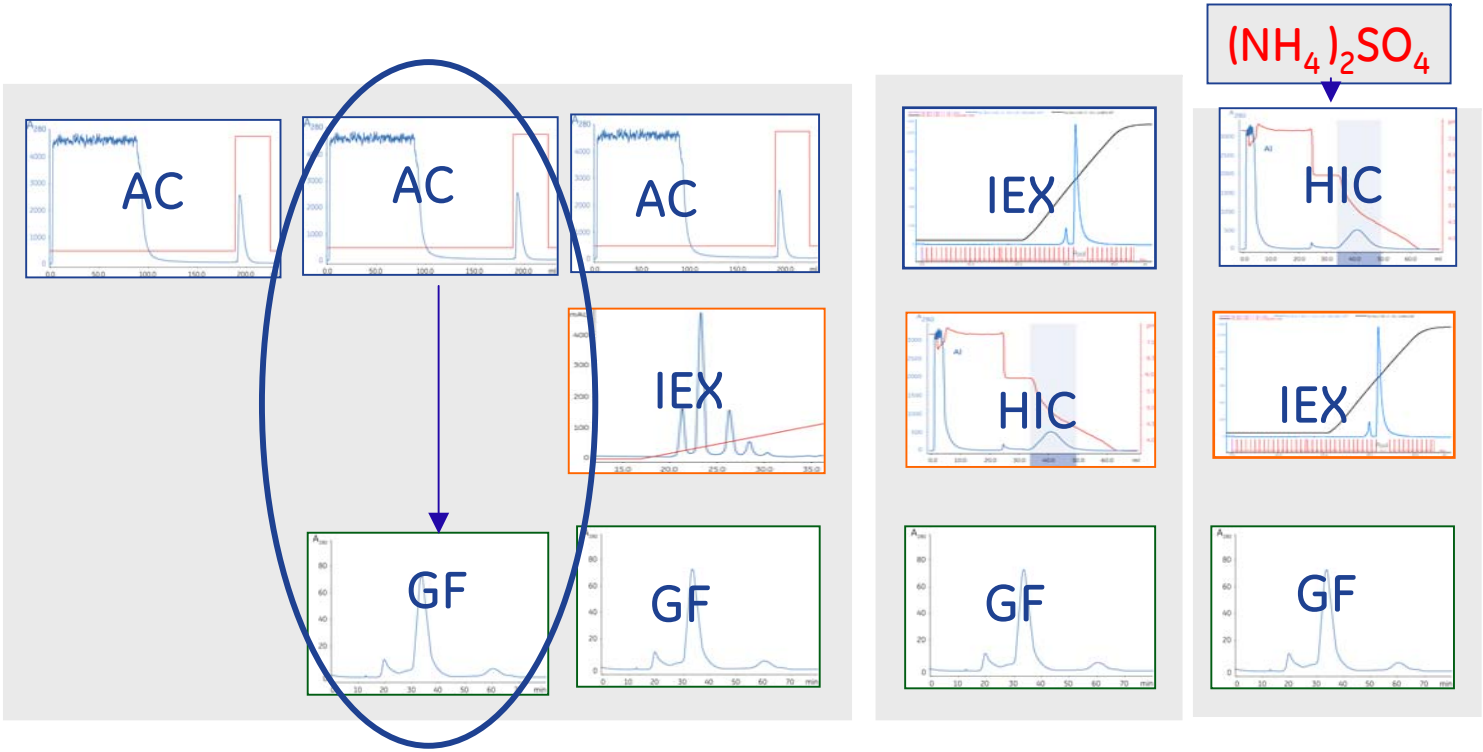
- Affinity + gel filtration
- Double tagging ⟶ affinity + affinity + gel filtration

层析技术的衔接

捕获

中度纯化

精细纯化

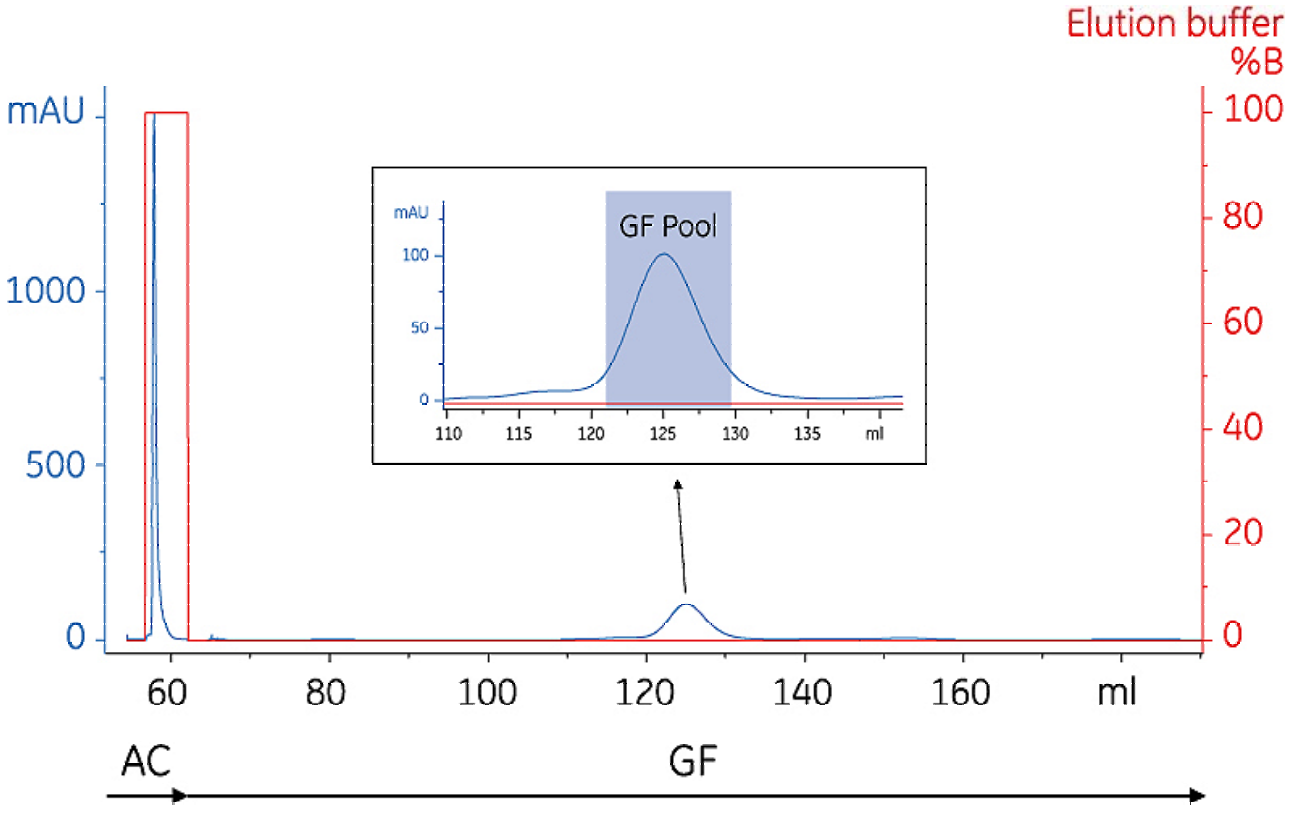


Proteins with known affinity ligand

- tagged proteins, antibodies

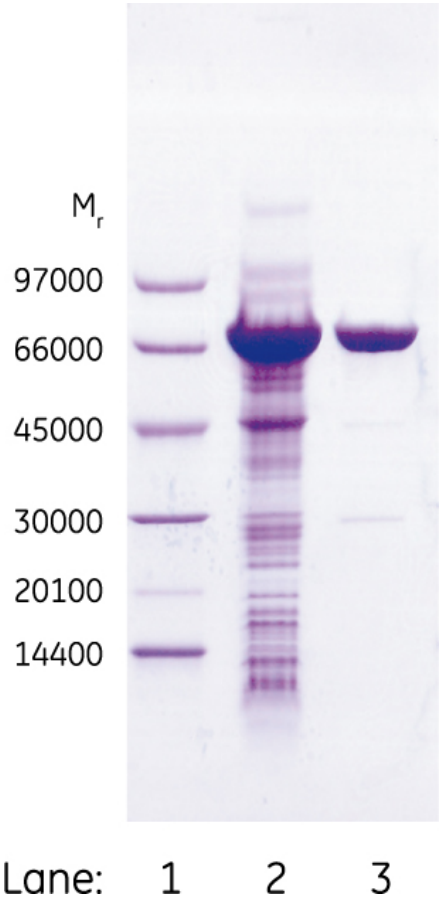
Affinity + gel filtration

Automated two-step purification on ÄKTAxpress™



MBPTrap™ HP

HiLoad™ 16/60 Superdex™ 200 pg



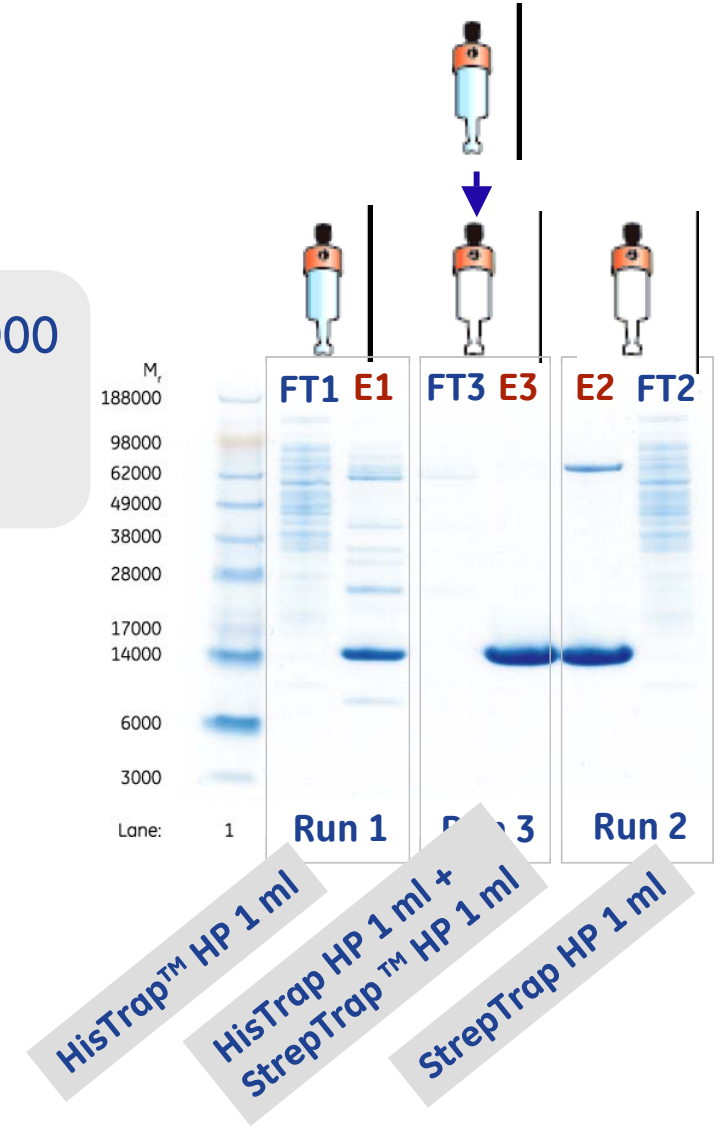
imagination at work

双标签得到全长蛋白

Protein: *Strep* tag II-Protein X-$(His)_6$ Mr ≈ 15 000
Sample: 15 ml *E. coli* lysate
System: ÄKTAxpress™

For two AC steps in series, check compatibility of start and end buffer conditions

e.g., *Strep tag* II tagged protein binds to StrepTactin™ Sepharose™ in presence of imidazole



Acknowledgement: Martina Nilsson, Biovitrum, Stockholm, Sweden

需要精细纯化步骤

Size heterogeneities

Aggregation

Proteolytic fragmentation

→ **use GF**

Charge heterogeneities

Heterogeniety in N- or C- terminus

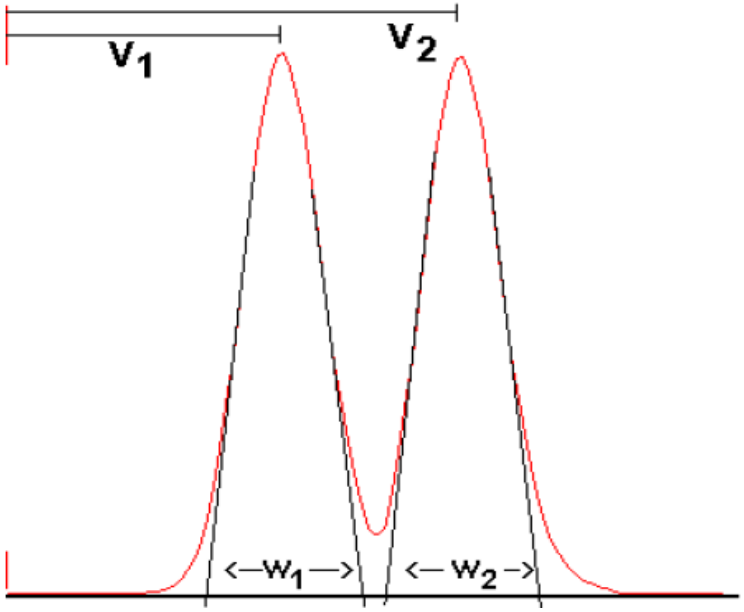
Post-translational modifications

e.g.phosphorylation, glycosylation etc.

→ **use IEX**

Content

How to get desired resolution?



$$R_s = \frac{V_2 - V_1}{(W_1 + W_2) / 2}$$

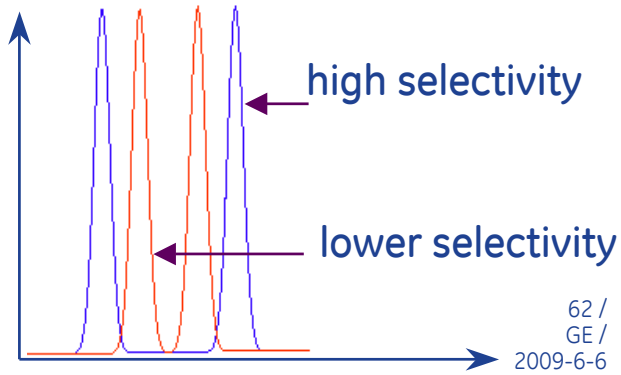
Selectivity or Efficiency ?

选择性 or 柱效？

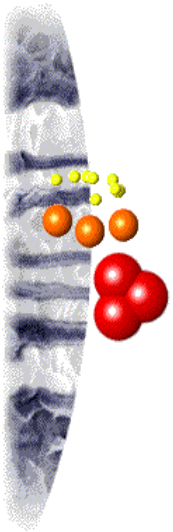
Selectivity 选择性

"Right" technologies and parameters to maximize the differences between proteins !

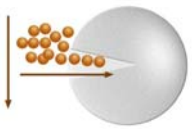
- Separation technologies 分离技术
- Chromatography media screen 层析填料筛选
- Separation conditions screen 分离条件筛选
- .....

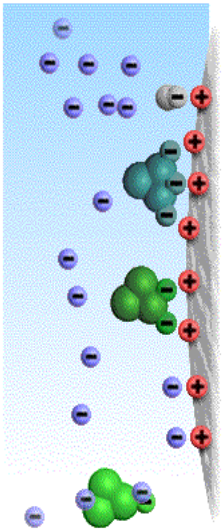


Separation technologies 分离技术

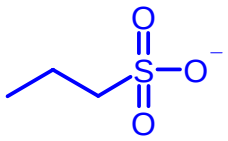


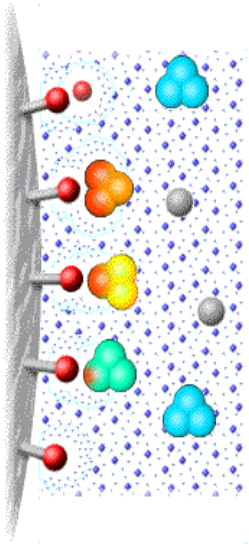
Gel filtration



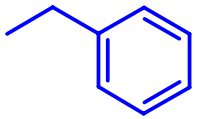


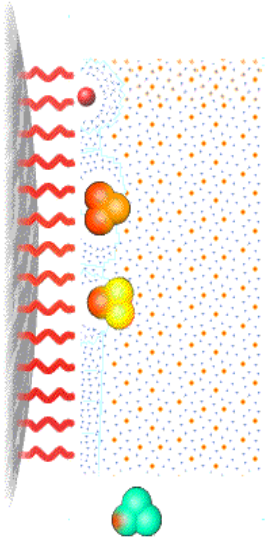
Ion exchange



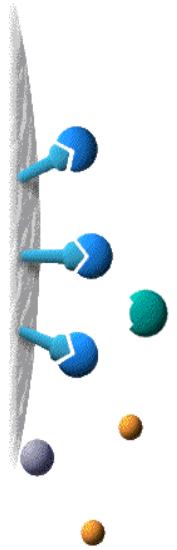


Hydrophobic interaction



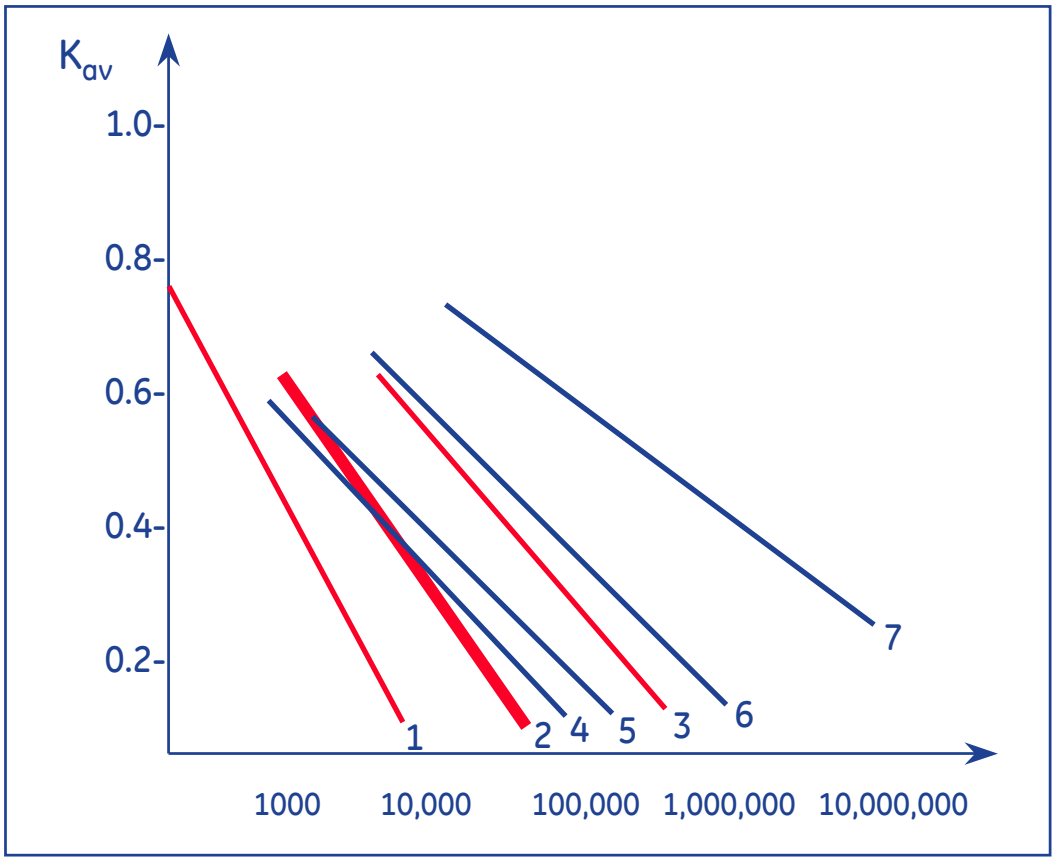


Reversed phase



Affinity

Separation media screen 层析填料筛选



1. Superdex™ Peptide
2. Superdex 75
3. Superdex 200
4. Sephacryl™ S-100 HR
5. Sephacryl S-200 HR
6. Sephacryl S-300 HR
7. Sephacryl S-400 HR

Superdex 75分离范围: 3,000~70,000 Da

Superdex 200分离范围: 10,000~600,000 Da

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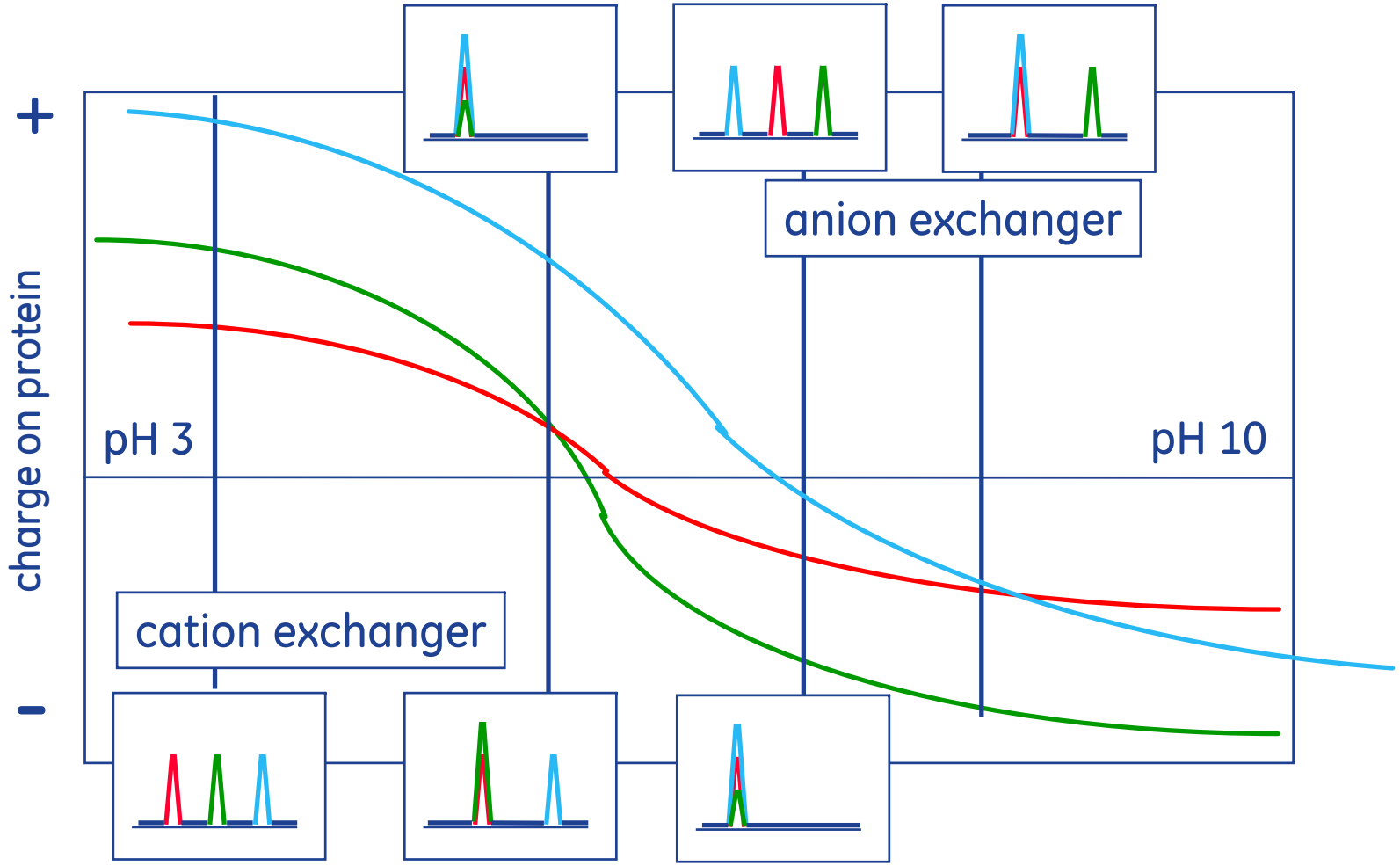
Separation conditions screen
分离条件筛选



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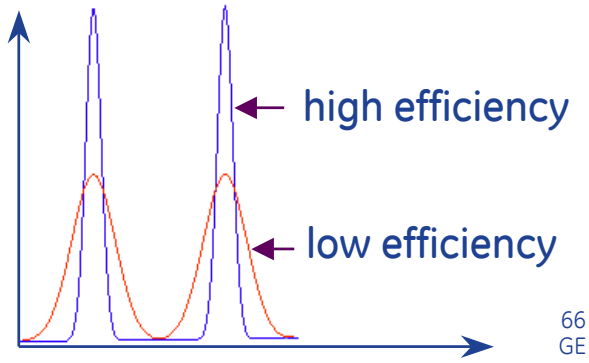
pH：关键参数

Efficiency 柱效

A powerful tool to improve Resolution !

- “Smaller” media v.s. back-pressure and scale-up
- Uniform media in size distribution
- Good packing or pre-packed column
- Lower flow rate to improve mass transfer



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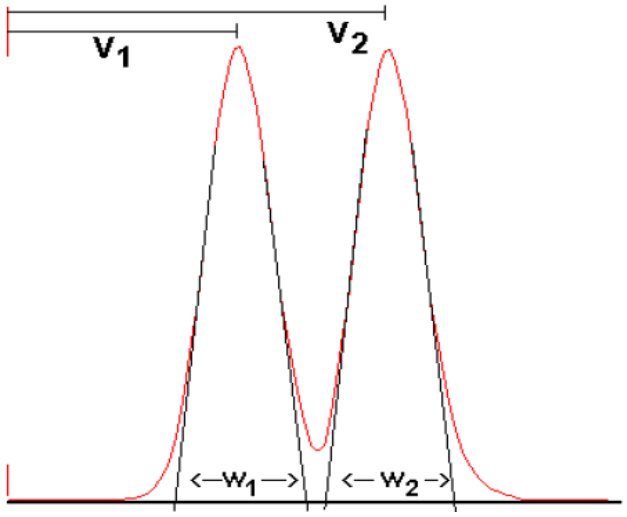
A **SUCCESSFUL** Purification 成功的纯化



$$R_s = \frac{V_2 - V_1}{(W_1 + W_2) / 2}$$

Selectivity 选择性:
- 实现有效分离的前提和基础!

Efficiency 柱效:
- 进一步提高分辨率的有力工具!

Content

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Summary of Strategies 总结

- 纯化前的考虑
  - 蛋白来源
  - 应用 (纯度要求)
  - 目标蛋白和杂质的性质
  - 特异性检测方法 (定性, 定量)
- 纯化策略
  - 策略1：纯化三步曲
  - 策略2：步骤越少收率越高
  - 策略3：交替运用互补层析技术, 合理衔接
  - 策略4：线性放大
- 成功的纯化SUCCESSFUL Purification
  - 选择性 or 柱效

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