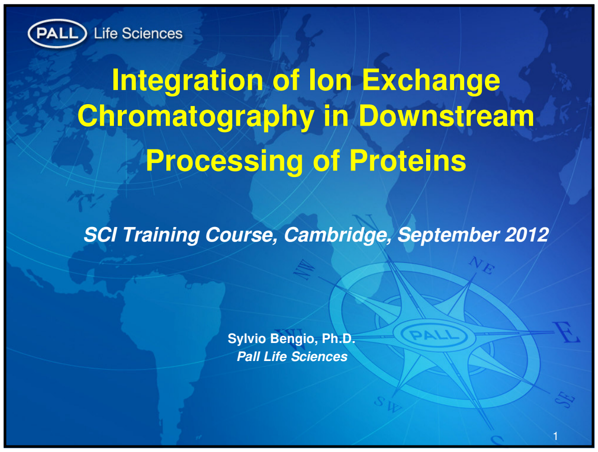

Integration of Ion Exchange Chromatography in Downstream Processing of Proteins

SCI Training Course, Cambridge, September 2012

Sylvio Bengio, Ph.D.
Pall Life Sciences

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Pall Chromatography Toolbox

Lab Scale Methods & Screening

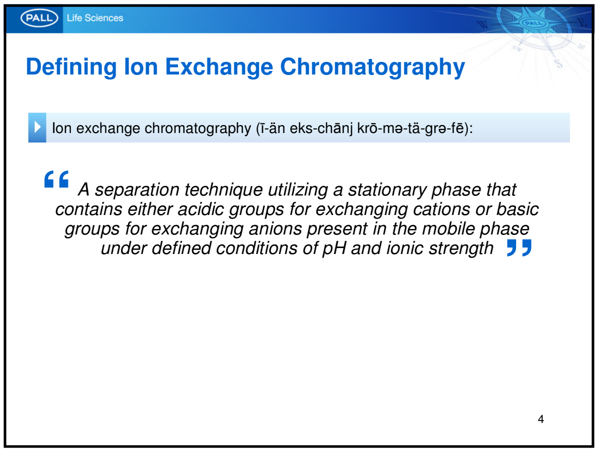
- AcroPrep™ and AcroWell™ filter plates,
  SELDI **Services** for sorbent screening
- 1 mL prepacked **PRC** columns
- Prepacked **AcroSep**™ columns
- **Mustang**® XT Acrodisc or XT5

Process Development

- Chromatography **sorbents**
- **Mustang** XT5 capsules
- Mustang XT Acrodisc
- 5 mL prepacked **PRC** columns
- Empty laboratory **LRC** glass

Scale-Up & Production

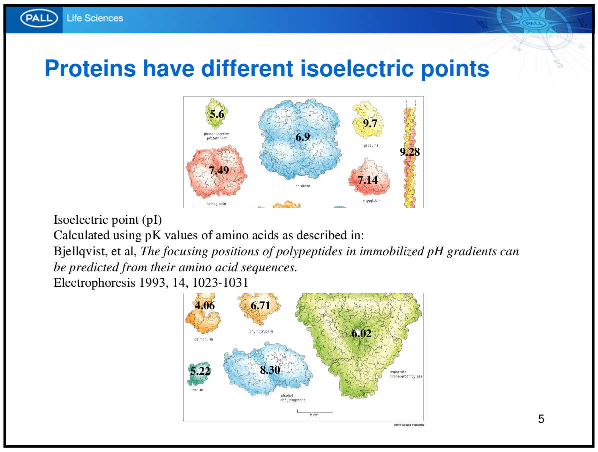
- Chromatography **sorbents**
- **Mustang** XT140 and XT5000 capsules
- **Resolute**® columns / Packing support
- **Packing stations**
- **PKP** and PK systems



Defining Ion Exchange Chromatography

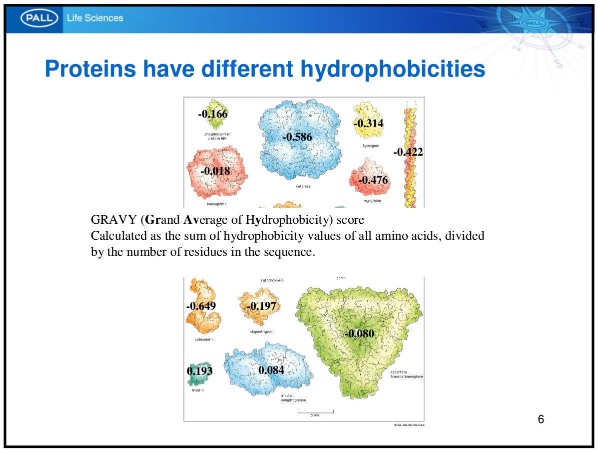
- Ion exchange chromatography (ī-ăn eks-chānj krō-mə-tă-grə-fē):

> “A separation technique utilizing a stationary phase that contains either acidic groups for exchanging cations or basic groups for exchanging anions present in the mobile phase under defined conditions of pH and ionic strength”



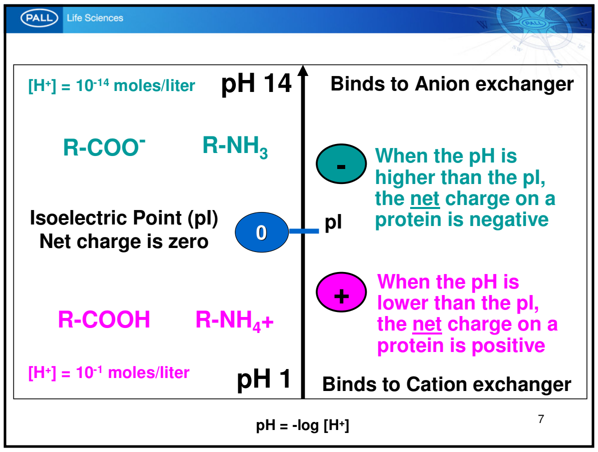
Proteins have different isoelectric points

Isoelectric point (pI)
Calculated using pK values of amino acids as described in:
Bjellqvist, et al, *The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences.*
Electrophoresis 1993, 14, 1023-1031



Proteins have different hydrophobicities

GRAVY (**Gr**and **A**verage of H**y**drophobicity) score
Calculated as the sum of hydrophobicity values of all amino acids, divided by the number of residues in the sequence.



$[H^+] = 10^{-14}$ moles/liter **pH 14**

Binds to Anion exchanger

$R\text{-}COO^-$ $R\text{-}NH_3$

- When the pH is higher than the pI, the <u>net</u> charge on a protein is negative

Isoelectric Point (pI)
Net charge is zero

0 pI

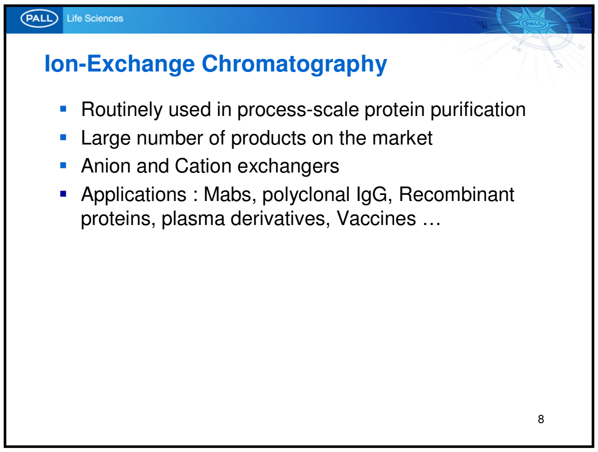
+ When the pH is lower than the pI, the <u>net</u> charge on a protein is positive

$R\text{-}COOH$ $R\text{-}NH_4+$

$[H^+] = 10^{-1}$ moles/liter **pH 1**

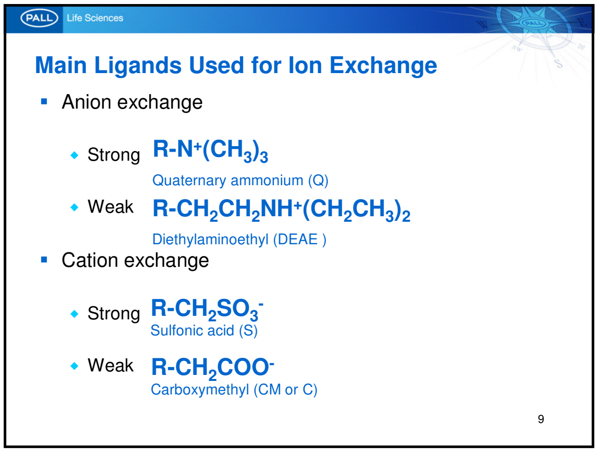
Binds to Cation exchanger

pH = -log [H+]



Ion-Exchange Chromatography

- Routinely used in process-scale protein purification
- Large number of products on the market
- Anion and Cation exchangers
- Applications : Mabs, polyclonal IgG, Recombinant proteins, plasma derivatives, Vaccines …

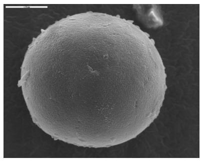


Main Ligands Used for Ion Exchange

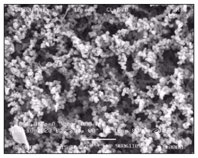
- Anion exchange
  - Strong $R\text{-}N^+(CH_3)_3$
    Quaternary ammonium (Q)
  - Weak $R\text{-}CH_2CH_2NH^+(CH_2CH_3)_2$
    Diethylaminoethyl (DEAE)
- Cation exchange
  - Strong $R\text{-}CH_2SO_3^-$
    Sulfonic acid (S)
  - Weak $R\text{-}CH_2COO^-$
    Carboxymethyl (CM or C)

Formats: Beads or Membrane adsorbers

Sorbents



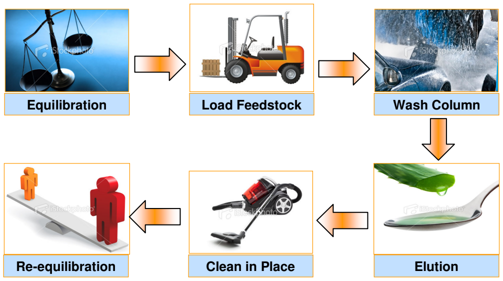
Membranes



« Salt Tolerant » Ion Exchange Ligands

- Vast majority of proteins acidic. Will be negatively charged around neutral pH or higher, anion exchange (Q, Deae) is more commonly used than cation (S, CM) for capture steps.
- Novel sorbents and membranes allow protein capture at moderate to high conductivities (e.g. >10 mS/cm) with high binding capacities.
  - For capture : direct load of feedstock
  - For polishing (flowthrough mode)
- Usually based on primary amine ligands

Typical Steps in Ion Exchange



Equilibration

Load Feedstock

Wash Column

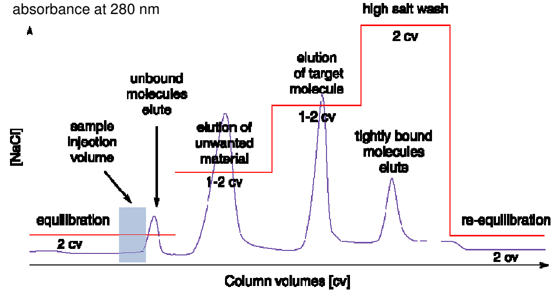
Elution

Clean in Place

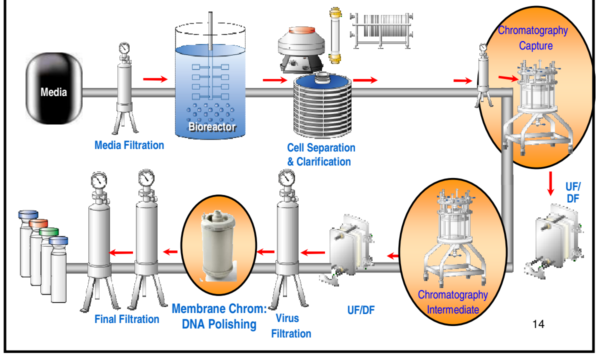
Re-equilibration

Ion Exchange Typical Chromatogram

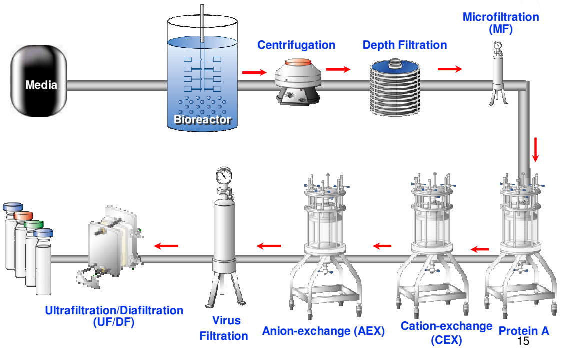
Proteins are monitored by UV absorbance at 280 nm



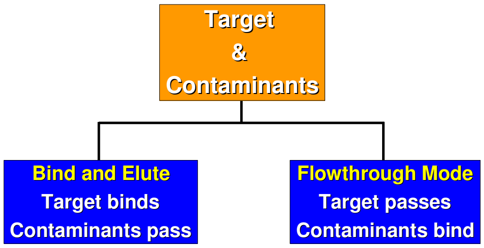
Chromatography in Process



Example: Typical Mab Platform Process



Bind/Elute and Flowthrough Modes



Target
&
Contaminants

Bind and Elute
Target binds
Contaminants pass

Flowthrough Mode
Target passes
Contaminants bind

Bind/Elute or Flowthrough mode ?

- Relative concentration of target
- Relative pI of target vs main impurity
- Feedstock volume
- Subsequent downstream process
- Target stability to process conditions
- Production facilities and equipment

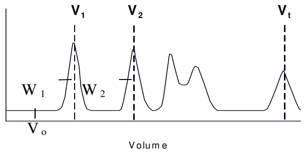
Selectivity and Resolution

Selectivity (α) **: ability of the matrix to separate peaks, i.e the distance between two peaks. Selectivity can vary from one sorbent to another for the same chemistry**

Expressed as :

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

with V0 : void volume of the column
V1 : elution volume of peak 1
V2 : elution volume of peak 2

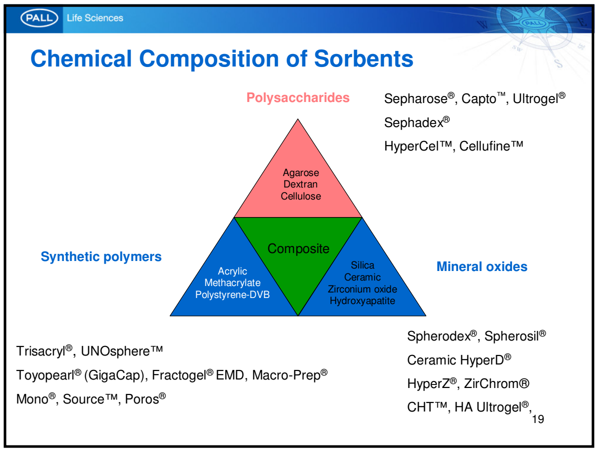


Resolution **: distance between peak maxima compared with the average base width of the 2 peaks. The further apart and the sharper the better the resolution.**

Expressed as :

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

with W1 : peak 1 width at half peak height
W2 : peak 2 width at half peak height



Chemical Composition of Sorbents

Polysaccharides

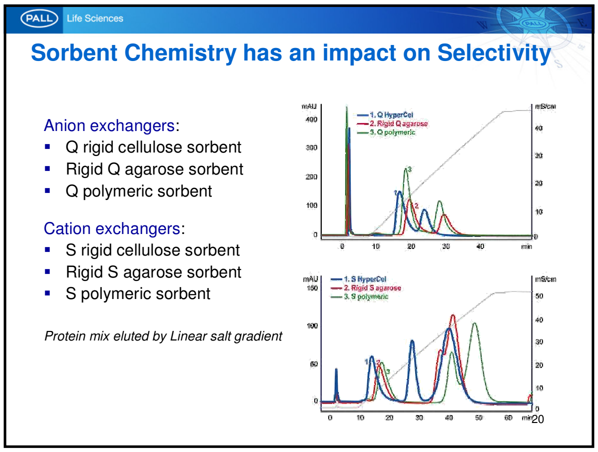
Sepharose®, Capto™, Ultrogel®
Sephadex®
HyperCel™, Cellufine™

Synthetic polymers

Mineral oxides

Trisacryl®, UNOsphere™
Toyopearl® (GigaCap), Fractogel® EMD, Macro-Prep®
Mono®, Source™, Poros®

Spherodex®, Spherosil®
Ceramic HyperD®
HyperZ®, ZirChrom®
CHT™, HA Ultrogel®,



Sorbent Chemistry has an impact on Selectivity

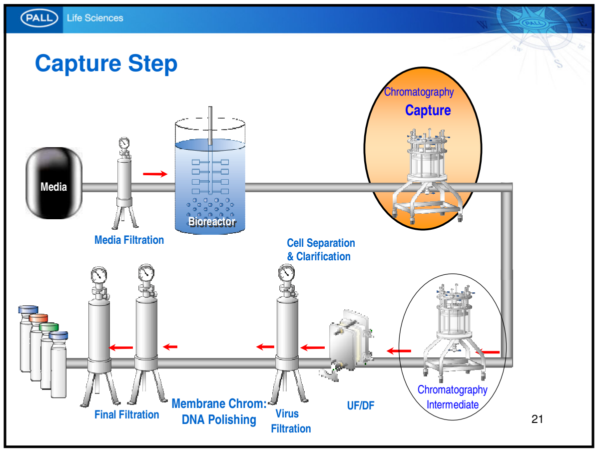
Anion exchangers:

- Q rigid cellulose sorbent
- Rigid Q agarose sorbent
- Q polymeric sorbent

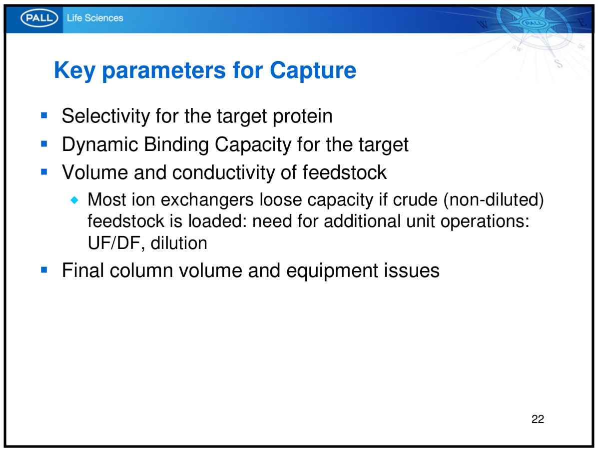
Cation exchangers:

- S rigid cellulose sorbent
- Rigid S agarose sorbent
- S polymeric sorbent

Protein mix eluted by Linear salt gradient

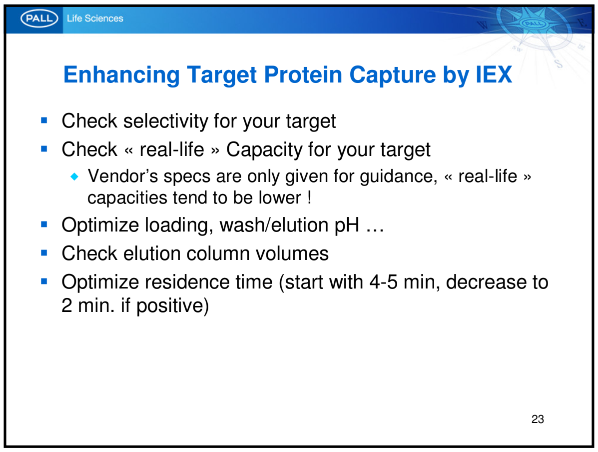


Capture Step



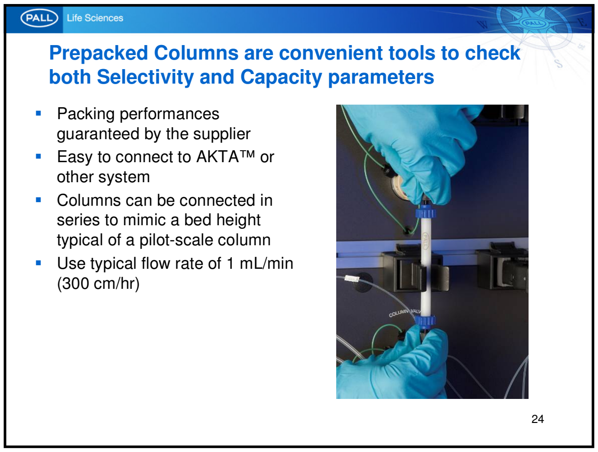
Key parameters for Capture

- Selectivity for the target protein
- Dynamic Binding Capacity for the target
- Volume and conductivity of feedstock
  - Most ion exchangers loose capacity if crude (non-diluted) feedstock is loaded: need for additional unit operations: UF/DF, dilution
- Final column volume and equipment issues



Enhancing Target Protein Capture by IEX

- Check selectivity for your target
- Check « real-life » Capacity for your target
  - Vendor’s specs are only given for guidance, « real-life » capacities tend to be lower !
- Optimize loading, wash/elution pH …
- Check elution column volumes
- Optimize residence time (start with 4-5 min, decrease to 2 min. if positive)



Prepacked Columns are convenient tools to check both Selectivity and Capacity parameters

- Packing performances guaranteed by the supplier
- Easy to connect to AKTA™ or other system
- Columns can be connected in series to mimic a bed height typical of a pilot-scale column
- Use typical flow rate of 1 mL/min (300 cm/hr)

Elution Conditions

- Elution can be achieved as usual by salt gradient.
- Elution may also be done by decreasing the pH. In this case, it allows to recover the product at lower conductivity allowing a direct load to an orthogonal ion exchanger or simplifying formulation

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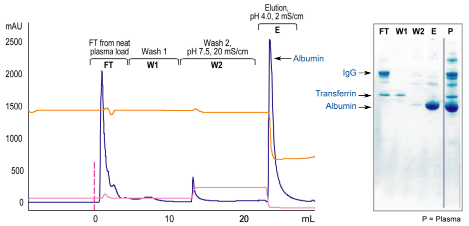
Ion Exchange for Capture : purification of HSA from crude, undiluted plasma



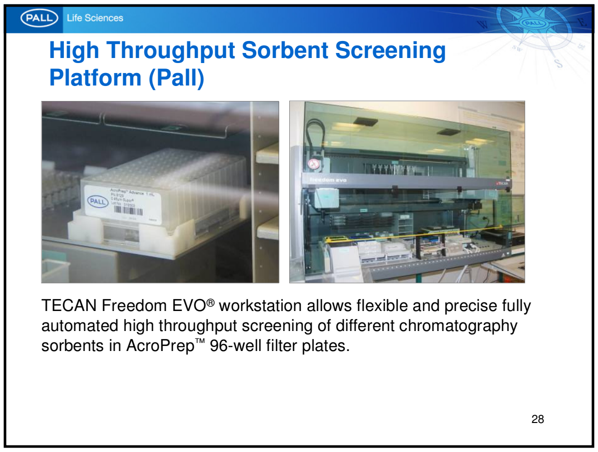
- Direct capture of HSA from undiluted feedstock: Purity > 99%, Yield ~90%
- Elution can be prompted by lowering pH only, no need to add NaCl, this allows a direct orthogonal load on S HyperCel cation exchange sorbent.

High Throughput Sorbent Screening

- **HTS methods** are well suited to quickly select the best sorbent for a specific step
- Saves time, sample and manpower
- Needed at early stages of process development
- Use 96 well plates combined with Design of Experiments (DoE) software tools

HTPD
HIGH-THROUGHPUT PROCESS DEVELOPMENT
FIRST INTERNATIONAL CONFERENCE
KRAKÓW, POLAND, OCTOBER 4–7, 2010

Second international conference: Avignon, June 4-7, 2012

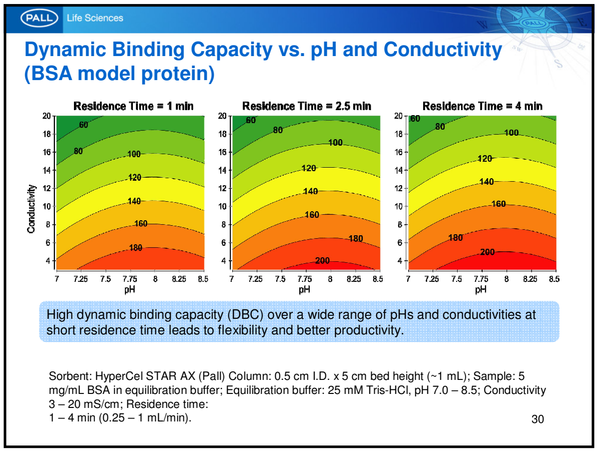


High Throughput Sorbent Screening Platform (Pall)

TECAN Freedom EVO® workstation allows flexible and precise fully automated high throughput screening of different chromatography sorbents in AcroPrep™ 96-well filter plates.

Capacity of Ion Exchange sorbents

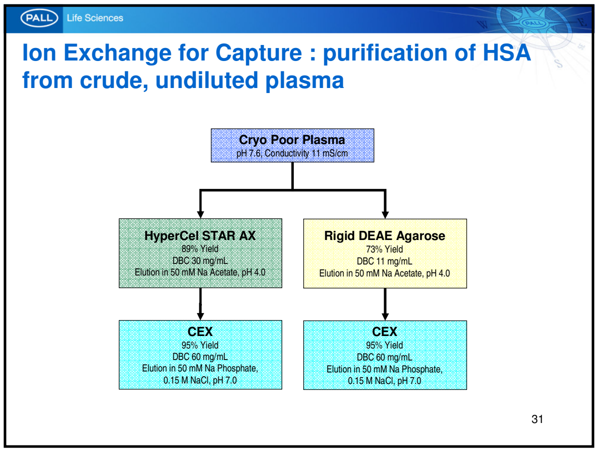
- Dynamic Binding Capacity (DBC) is a key parameter in process chromatography.
  - To maximize productivity during capture steps, with feedstream having high expression titers (e.g. Monoclonal antibodies 5-10 g/L)
- DBC varies according to the sorbent type, the linear flow rate or residence time
- pH and ionic strength of the feedstock also impact DBC.
  - New generation of « salt tolerant » sorbents



Dynamic Binding Capacity vs. pH and Conductivity (BSA model protein)

High dynamic binding capacity (DBC) over a wide range of pHs and conductivities at short residence time leads to flexibility and better productivity.

Sorbent: HyperCel STAR AX (Pall) Column: 0.5 cm I.D. x 5 cm bed height (~1 mL); Sample: 5 mg/mL BSA in equilibration buffer; Equilibration buffer: 25 mM Tris-HCl, pH 7.0 – 8.5; Conductivity 3 – 20 mS/cm; Residence time:
1 – 4 min (0.25 – 1 mL/min).



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Ion Exchange for Capture : purification of HSA from crude, undiluted plasma

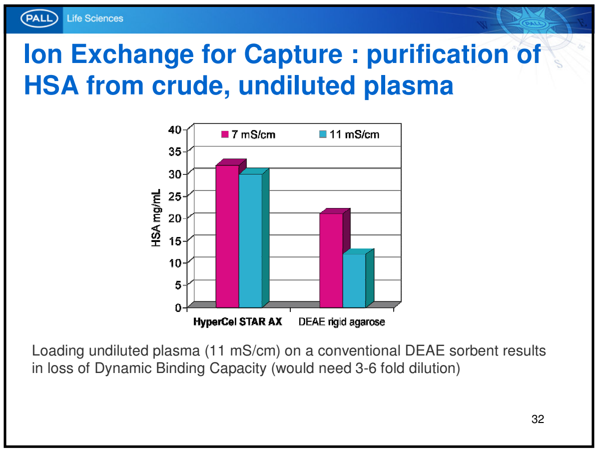
Cryo Poor Plasma
pH 7.6, Conductivity 11 mS/cm

HyperCel STAR AX
89% Yield
DBC 30 mg/mL
Elution in 50 mM Na Acetate, pH 4.0

Rigid DEAE Agarose
73% Yield
DBC 11 mg/mL
Elution in 50 mM Na Acetate, pH 4.0

CEX
95% Yield
DBC 60 mg/mL
Elution in 50 mM Na Phosphate, 0.15 M NaCl, pH 7.0

CEX
95% Yield
DBC 60 mg/mL
Elution in 50 mM Na Phosphate, 0.15 M NaCl, pH 7.0



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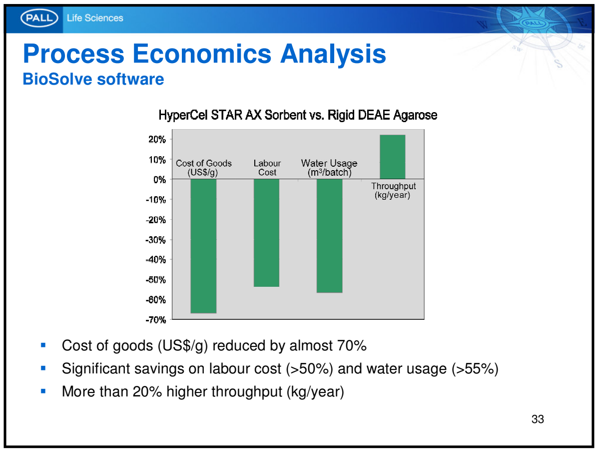
Ion Exchange for Capture : purification of HSA from crude, undiluted plasma

Loading undiluted plasma (11 mS/cm) on a conventional DEAE sorbent results in loss of Dynamic Binding Capacity (would need 3-6 fold dilution)



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Process Economics Analysis

BioSolve software

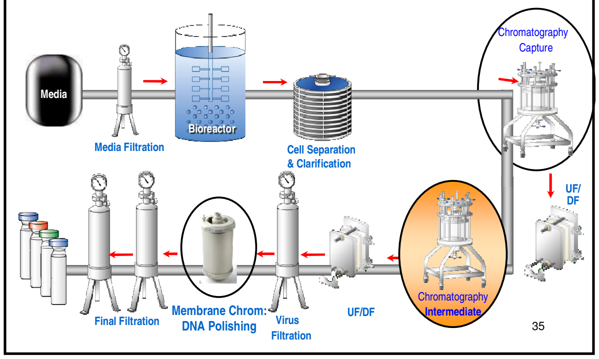
HyperCel STAR AX Sorbent vs. Rigid DEAE Agarose

- Cost of goods (US$/g) reduced by almost 70%
- Significant savings on labour cost (>50%) and water usage (>55%)
- More than 20% higher throughput (kg/year)

Ion Exchange at Capture Step: Conclusions

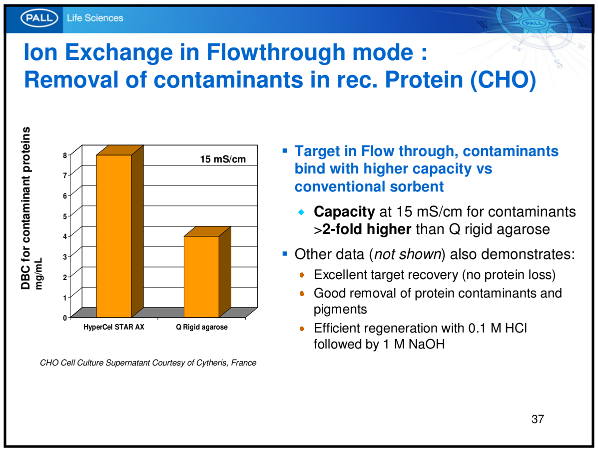
- Check <u>selectivity for the target</u> first
- Choose sorbent that maximizes **Dynamic Binding Capacity** with minimal feed pretreatment (UF/DF, dilution)
- Consider re-equilibration duration, column volumes needed for elution and cleaning in place regimes
- Consider column final volume at production scale and equipment costs

Intermediate Step



Key parameters for Intermediate step

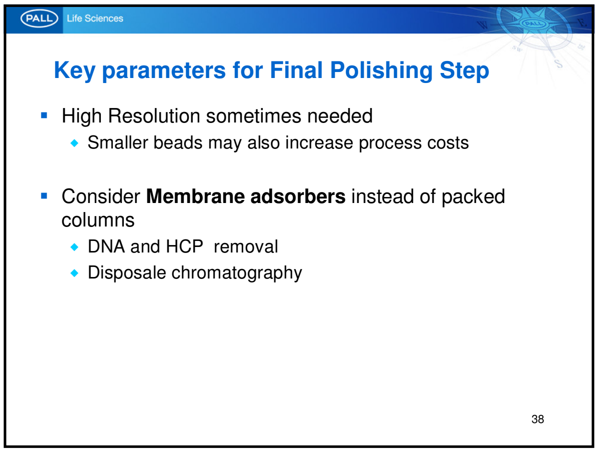
- Selectivity for the target protein vs main contaminants
  - e.g. Host Cell Proteins, aggregates, misfolds….
- Consider **Flowthrough mode** (contaminants bind)
- High resolution steps may help in some cases
  - Smaller beads may also increase process costs



Ion Exchange in Flowthrough mode : Removal of contaminants in rec. Protein (CHO)

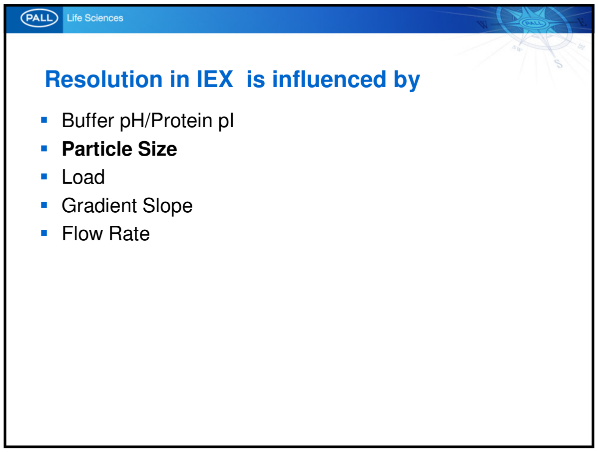
CHO Cell Culture Supernatant Courtesy of Cytheris, France

- **Target in Flow through, contaminants bind with higher capacity vs conventional sorbent**
  - **Capacity** at 15 mS/cm for contaminants >**2-fold higher** than Q rigid agarose
- Other data (*not shown*) also demonstrates:
  - Excellent target recovery (no protein loss)
  - Good removal of protein contaminants and pigments
  - Efficient regeneration with 0.1 M HCl followed by 1 M NaOH



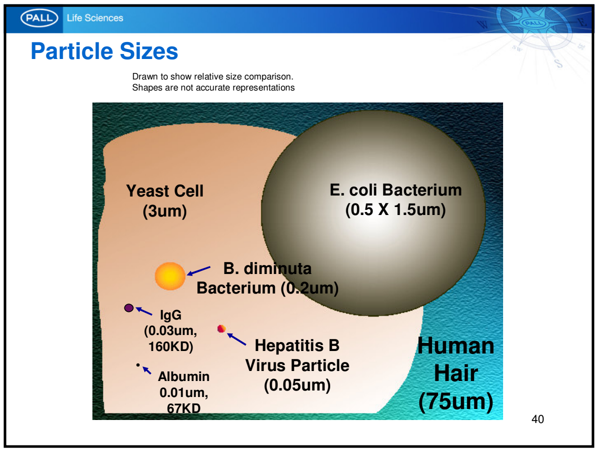
Key parameters for Final Polishing Step

- High Resolution sometimes needed
  - Smaller beads may also increase process costs
- Consider **Membrane adsorbers** instead of packed columns
  - DNA and HCP removal
  - Disposale chromatography



Resolution in IEX is influenced by

- Buffer pH/Protein pI
- **Particle Size**
- Load
- Gradient Slope
- Flow Rate



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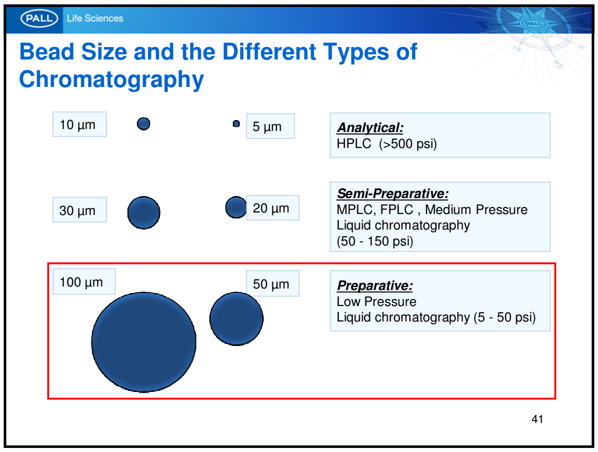
Particle Sizes

Drawn to show relative size comparison.
Shapes are not accurate representations



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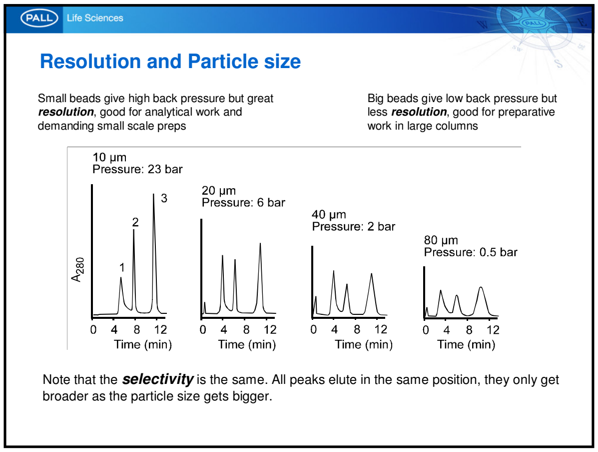
Bead Size and the Different Types of Chromatography

Analytical:
HPLC (>500 psi)

Semi-Preparative:
MPLC, FPLC , Medium Pressure
Liquid chromatography
(50 - 150 psi)

Preparative:
Low Pressure
Liquid chromatography (5 - 50 psi)



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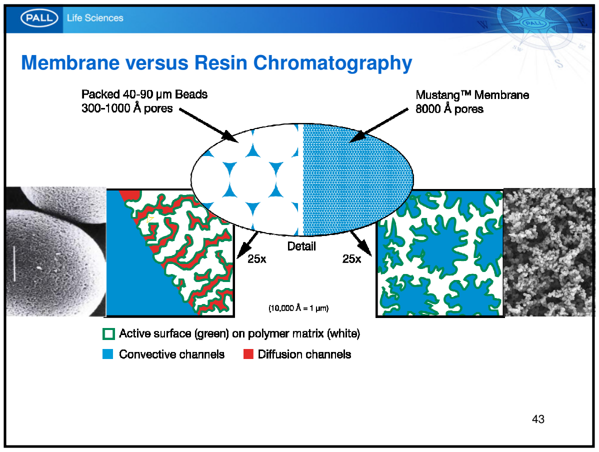
Resolution and Particle size

Small beads give high back pressure but great ***resolution***, good for analytical work and demanding small scale preps

Big beads give low back pressure but less ***resolution***, good for preparative work in large columns

Note that the ***selectivity*** is the same. All peaks elute in the same position, they only get broader as the particle size gets bigger.



Membrane versus Resin Chromatography

Packed 40-90 µm Beads
300-1000 Å pores

Mustang™ Membrane
8000 Å pores

Detail

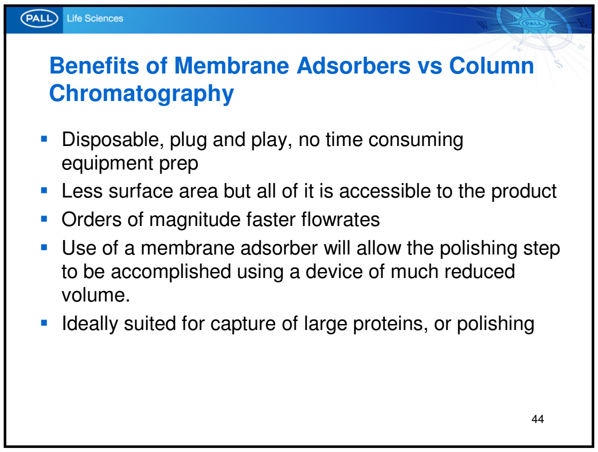
25x

25x

(10,000 Å = 1 µm)

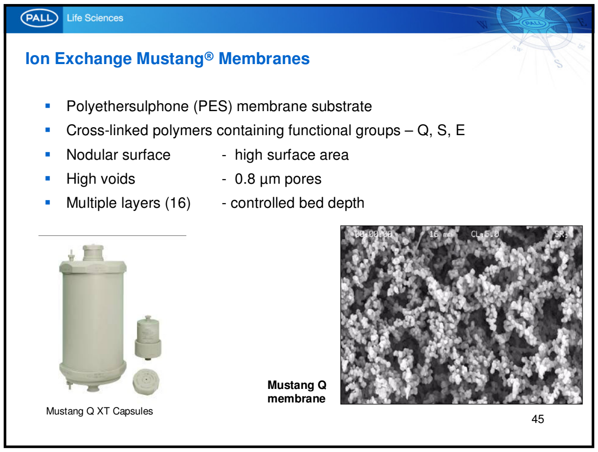
☐ Active surface (green) on polymer matrix (white)

🟦 Convective channels 🟥 Diffusion channels



Benefits of Membrane Adsorbers vs Column Chromatography

- Disposable, plug and play, no time consuming equipment prep
- Less surface area but all of it is accessible to the product
- Orders of magnitude faster flowrates
- Use of a membrane adsorber will allow the polishing step to be accomplished using a device of much reduced volume.
- Ideally suited for capture of large proteins, or polishing

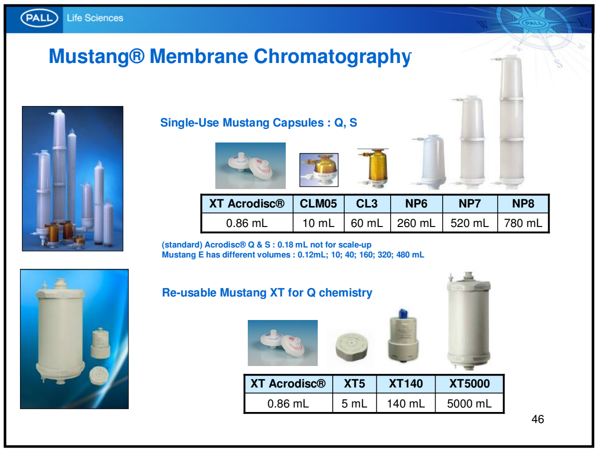


Ion Exchange Mustang® Membranes

- Polyethersulphone (PES) membrane substrate
- Cross-linked polymers containing functional groups – Q, S, E
- Nodular surface - high surface area
- High voids - 0.8 µm pores
- Multiple layers (16) - controlled bed depth

Mustang Q XT Capsules

Mustang Q membrane



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Mustang® Membrane Chromatography

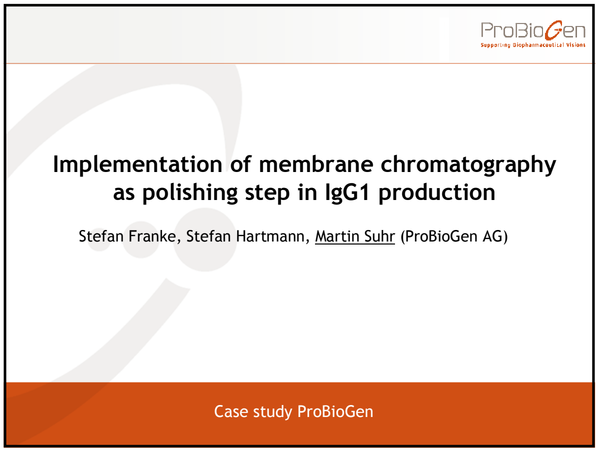
Single-Use Mustang Capsules : Q, S

XT Acrodisc®	CLM05	CL3	NP6	NP7	NP8
0.86 mL	10 mL	60 mL	260 mL	520 mL	780 mL

(standard) Acrodisc® Q & S : 0.18 mL not for scale-up
Mustang E has different volumes : 0.12mL; 10; 40; 160; 320; 480 mL

Re-usable Mustang XT for Q chemistry

XT Acrodisc®	XT5	XT140	XT5000
0.86 mL	5 mL	140 mL	5000 mL

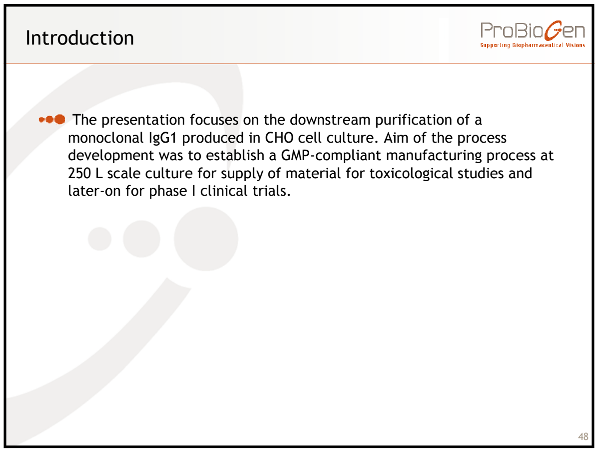


ProBioGen
Supporting Biopharmaceutical Visions

Implementation of membrane chromatography as polishing step in IgG1 production

Stefan Franke, Stefan Hartmann, Martin Suhr (ProBioGen AG)

Case study ProBioGen



Introduction

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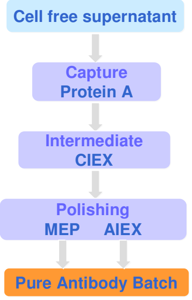
- The presentation focuses on the downstream purification of a monoclonal IgG1 produced in CHO cell culture. Aim of the process development was to establish a GMP-compliant manufacturing process at 250 L scale culture for supply of material for toxicological studies and later-on for phase I clinical trials.

Outline

I. Evaluation of mixed-mode chromatography as a polishing step for the removal of MAb aggregates.
II. *Implementation of membrane chromatography as a polishing step for host cell DNA removal: from development to production.*
III. Removal of HCP during the membrane chromatography step.
IV. Pilot-scale study of viral clearance.

Human IgG1 from CHO culture – DSP Process

- Three basic chromatography steps.
- Two virus depletion steps: low pH treatment and nanofiltration.
- Polishing (DNA and HCP removal) - Two techniques were investigated:
  - MEP HyperCel™ mixed-mode chromatography for aggregate removal.
  - **Anion exchange Mustang® Q chromatography membrane adsorber (AIEX) used in negative-mode**

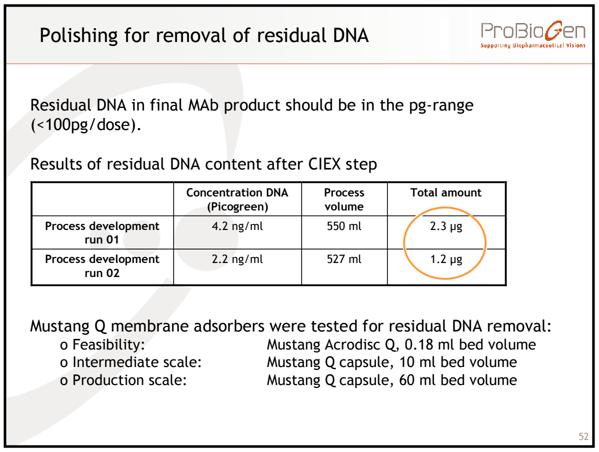


Material and Methods

Material:
IgG1 was expressed in serum-free CHO cell culture (fed-batch).

Methods:
All chromatography steps were performed using ÄKTA™ systems (GE HealthCare).
MEP HyperCel™ sorbent and Mustang® Q membrane adsorbers were supplied by Pall.

Analytics:
o IgG1 purity: Size Exclusion Chromatography
oResidual DNA: Picogreen Assay (Invitrogen) or quantitative PCR.
o Residual HCP: generic anti CHO-HCP-ELISA (Cygnus Technologies).
o Virus spiking study: external service provider.



Polishing for removal of residual DNA

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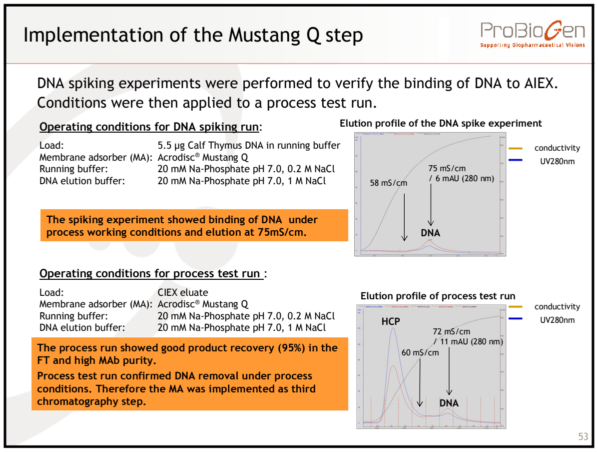
Residual DNA in final MAb product should be in the pg-range (<100pg/dose).

Results of residual DNA content after CIEX step

	Concentration DNA (Picogreen)	Process volume	Total amount
Process development run 01	4.2 ng/ml	550 ml	2.3 µg
Process development run 02	2.2 ng/ml	527 ml	1.2 µg

Mustang Q membrane adsorbers were tested for residual DNA removal:

o Feasibility:	Mustang Acrodisc Q, 0.18 ml bed volume
o Intermediate scale:	Mustang Q capsule, 10 ml bed volume
o Production scale:	Mustang Q capsule, 60 ml bed volume



Implementation of the Mustang Q step

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DNA spiking experiments were performed to verify the binding of DNA to AIEX. Conditions were then applied to a process test run.

Operating conditions for DNA spiking run:

Load:	5.5 µg Calf Thymus DNA in running buffer
Membrane adsorber (MA):	Acrodisc® Mustang Q
Running buffer:	20 mM Na-Phosphate pH 7.0, 0.2 M NaCl
DNA elution buffer:	20 mM Na-Phosphate pH 7.0, 1 M NaCl

The spiking experiment showed binding of DNA under process working conditions and elution at 75mS/cm.

Elution profile of the DNA spike experiment

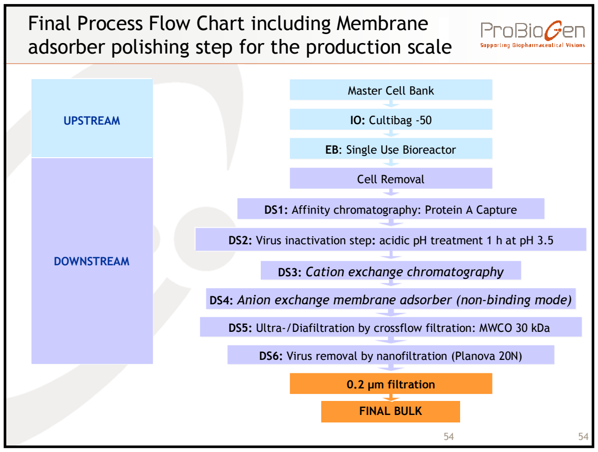
Operating conditions for process test run :

Load:	CIEX eluate
Membrane adsorber (MA):	Acrodisc® Mustang Q
Running buffer:	20 mM Na-Phosphate pH 7.0, 0.2 M NaCl
DNA elution buffer:	20 mM Na-Phosphate pH 7.0, 1 M NaCl

The process run showed good product recovery (95%) in the FT and high MAb purity.

Process test run confirmed DNA removal under process conditions. Therefore the MA was implemented as third chromatography step.

Elution profile of process test run



Final Process Flow Chart including Membrane adsorber polishing step for the production scale

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Master Cell Bank

IO: Cultibag -50

EB: Single Use Bioreactor

Cell Removal

DS1: Affinity chromatography: Protein A Capture

DS2: Virus inactivation step: acidic pH treatment 1 h at pH 3.5

DS3: *Cation exchange chromatography*

DS4: *Anion exchange membrane adsorber (non-binding mode)*

DS5: Ultra-/Diafiltration by crossflow filtration: MWCO 30 kDa

DS6: Virus removal by nanofiltration (Planova 20N)

0.2 µm filtration

FINAL BULK

IgG1 recovery after Mustang Q Polishing

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Unit:	Scale dependent
Buffer A:	20 mM Na-Phosphate pH 7.0
Buffer B (optional for eluting):	20 mM Na-Phosphate pH 7.0, 1 M NaCl
Conditioning of load:	1:2 dilution of the CIEX eluate with Buffer A

The 10 ml Mustang Q capsule was selected for production at the 50 L scale, and the 60 ml capsule for 250 L.

Run	Process Volume (mL)	Mustang Q unit	Conductivity	IgG1 concentration (UV280nm)	Protein Load (g product/ml bed volume)	MAb recovery
Consistency run 02	1010	10 mL Capsule	11.3 mS/cm	1.7	n/a	98.5 %
250 L Run	37600	60 mL Capsule	12.9 mS/cm	1.7	1.07	96.1 %
50 L Run	5609	10 mL Capsule	11.8 mS/cm	2.4	1.34	97.9 %
250 L GMP run	40150	60 mL Capsule	12.5 mS/cm	1.7	1.14	104.2 %

Data showed high IgG1 recovery (> 96 %) independent of the scale applied.

Process conditions of the membrane adsorber for production

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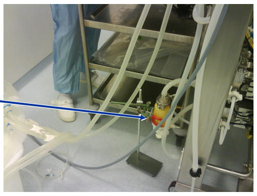
	Tox 1	Tox 2	GMP
Scale	250 L	50 L	250 L
Process volume	225 L	44 L	238 L
Process conditions	Mustang Q capsule MV: 60 mL Flow rate: 36 L/h (10 MV/min)*	Mustang Q capsule MV: 10 mL Flow rate: 30 ml/min** (3 MV/min)*	Mustang Q capsule MV: 60 mL Flow rate: 36 L/h (10 MV/min)*

*MV: membrane volume according supplier specification
**: due to technical reasons as pressure maximum was reached



Mustang Q 60 mL Capsule

Impurities: Removal of residual DNA

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Results of Host Cell DNA Depletion (Picogreen Assay)

	Development Run 1 (10 L Scale)		Development Run 2 (10 L Scale)	
Process Step	DNA ng/ml	Product mg/ml	DNA ng/ml	Product mg/ml
Cell culture supernatant	5951	n/a	7538	n/a
After 1st chromatography (Protein A)	19	6.6	22.2	7.2
After 2nd chromatography step (CIEX)	4.2	3.4	2.2	3.4

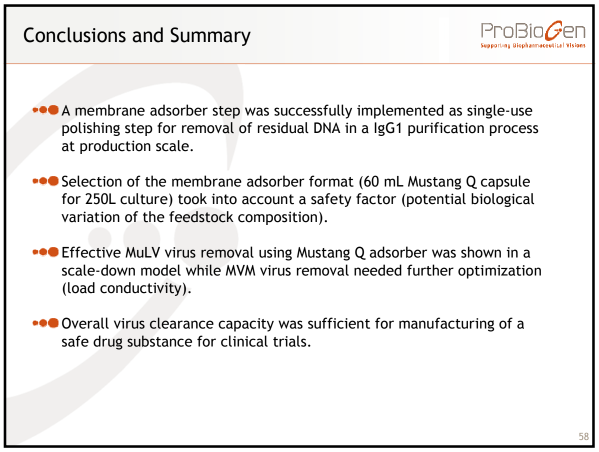
After CIEX the DNA content was already in the low ng range. The Picogreen Assay could not applied for analysis of AIEX samples so samples were analyzed externally using a qualified qPCR method (assay sensitivity < 1pg).

Results of Host Cell DNA data determined by qPCR

Sample/Process step	Tox - 250 L	Tox - 50 L	GMP - 250 L
Final bulk	< 40 pg/ml*	< 17 pg/ml*	< 16 pg/ml*

*: below lower limit of quantification of the assay (LLOQ)

The membrane adsorber step showed effective removal of residual DNA at production scale.



Conclusions and Summary

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- A membrane adsorber step was successfully implemented as single-use polishing step for removal of residual DNA in a IgG1 purification process at production scale.
- Selection of the membrane adsorber format (60 mL Mustang Q capsule for 250L culture) took into account a safety factor (potential biological variation of the feedstock composition).
- Effective MuLV virus removal using Mustang Q adsorber was shown in a scale-down model while MVM virus removal needed further optimization (load conductivity).
- Overall virus clearance capacity was sufficient for manufacturing of a safe drug substance for clinical trials.

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Thank You !

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