UNIVERSITY OF CAMBRIDGE

Protein Purification Part 2

Day 4: Thursday 23rd March

Continuing from Yesterday

- In Part 1 we learnt about:
  - Lysis techniques
  - Affinity chromatography including affinity tags and resins

- In Part 2 we will learn about:
  - Other chromatography techniques
    - Size-exclusion
    - Ion exchange
    - HIC and others
  - Chromatography equipment
    - The dos and don’ts of good chromatography

Why might you need to do a 2-step purification?

- Isn’t my protein pure enough after affinity chromatography?

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- Isn’t my protein pure enough after affinity chromatography?

- Possibly not, other protein contaminants can interfere with subsequent experiments
  - Tomorrow we’ll discuss how to evaluate this

Why might you need to do a 2-step purification?

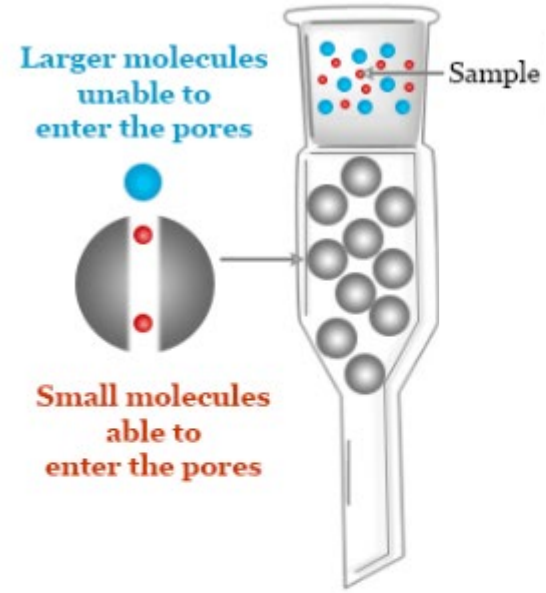
- Isn’t my protein pure enough after affinity chromatography?

- Possibly not, other protein contaminants can interfere with subsequent experiments
  - Tomorrow we’ll discuss how to evaluate this

- Also, there might be components in our buffer we need to remove
  - Although dialysis is one approach, 2-step purification is another

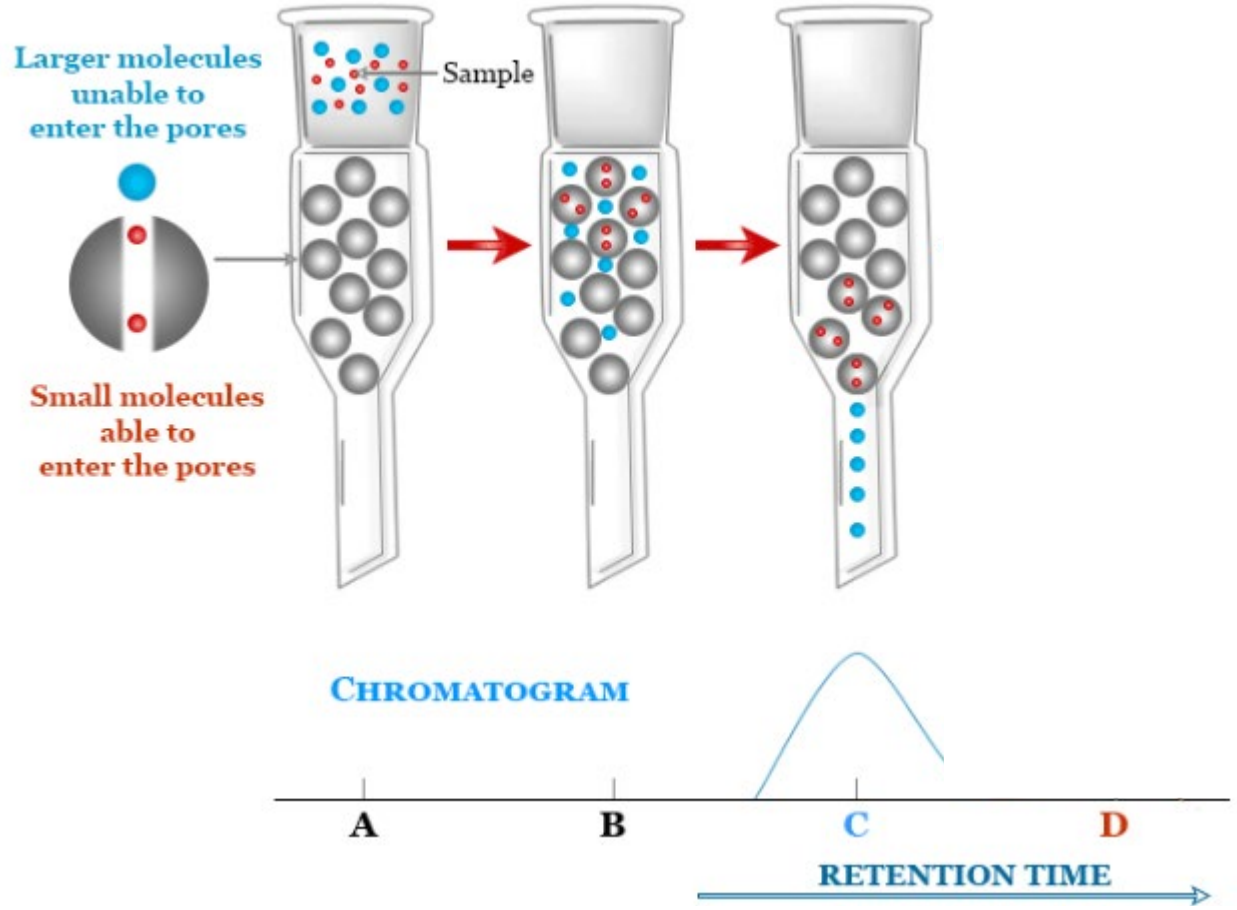
Size Exclusion Chromatography

- Separates based on size



Size Exclusion Chromatography

- Separates based on size
- Large proteins elute first
- Smaller ones are trapped in the resin and elute later



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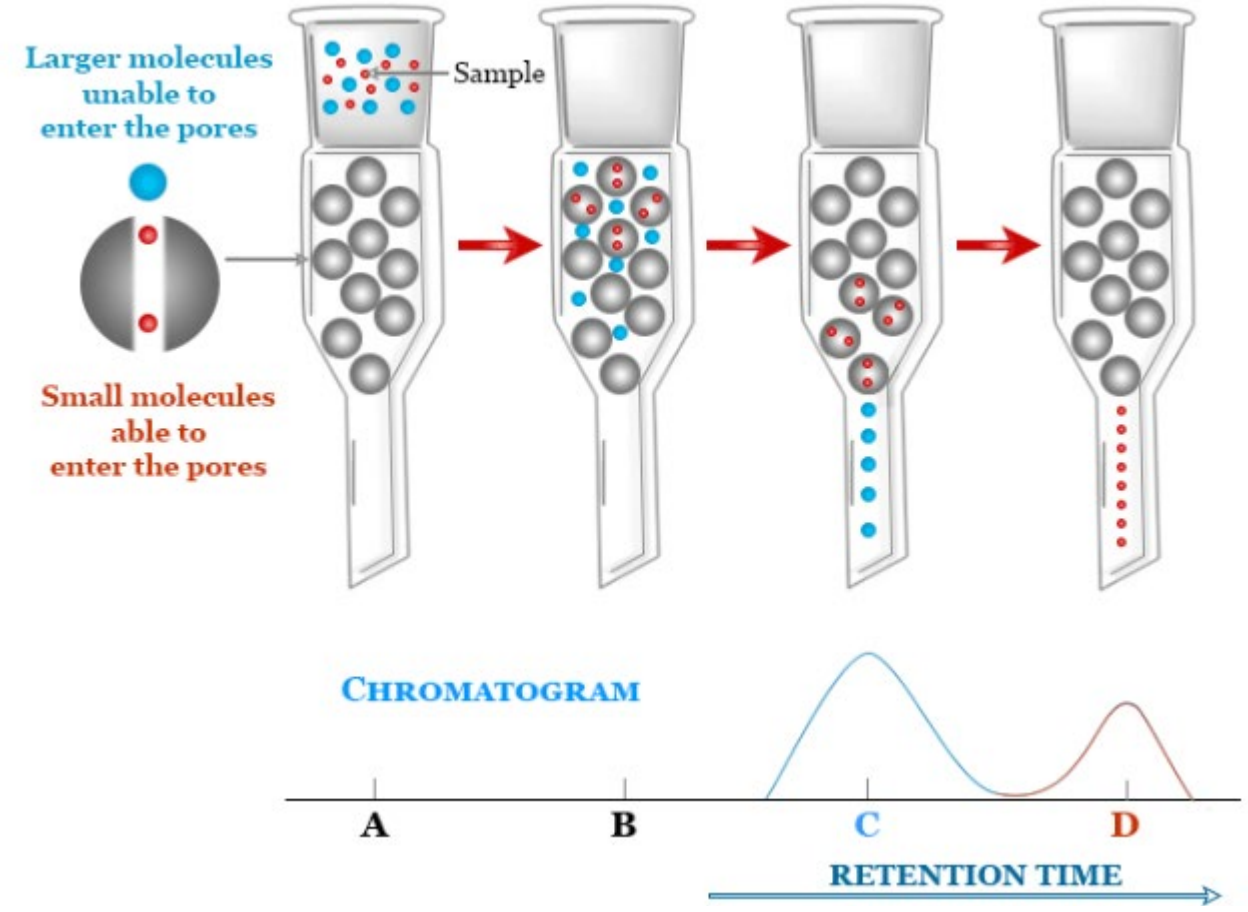
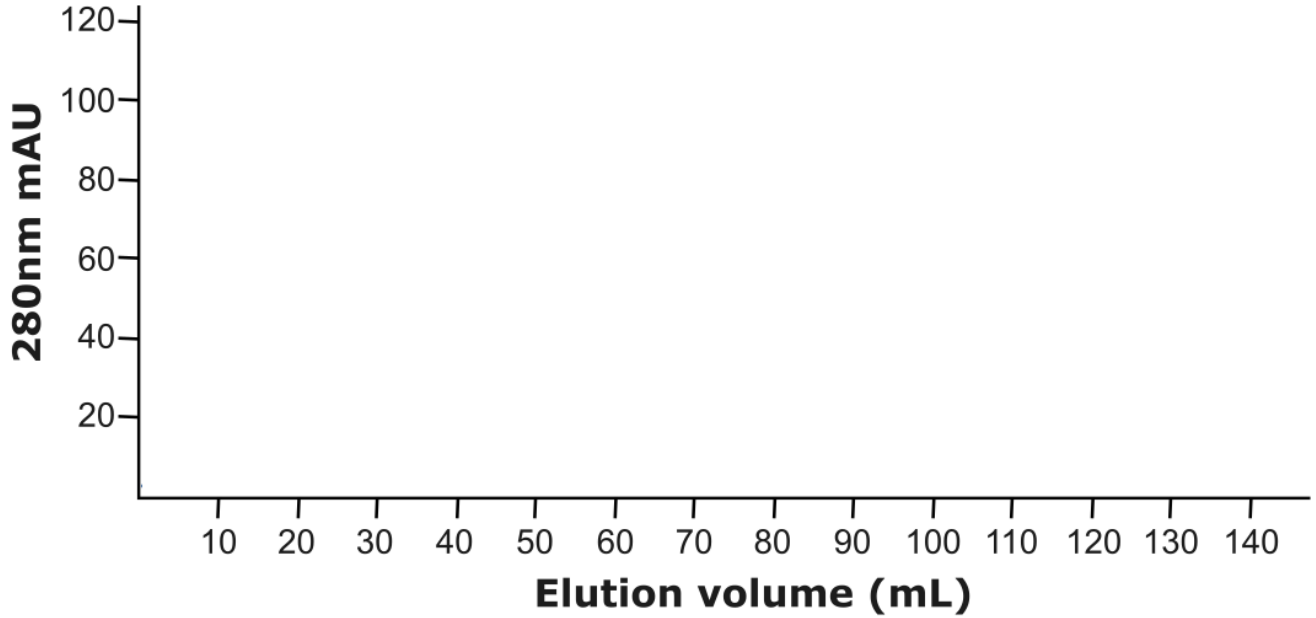


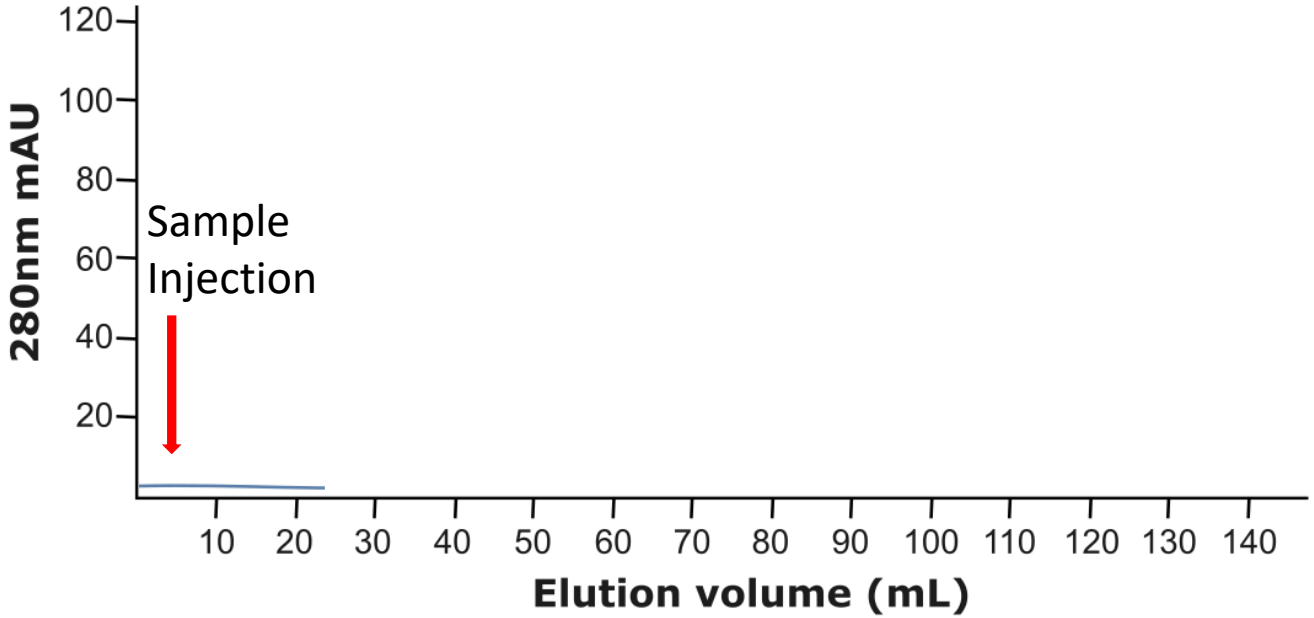
Monitoring Size Exclusion Chromatography

- The chromatogram:



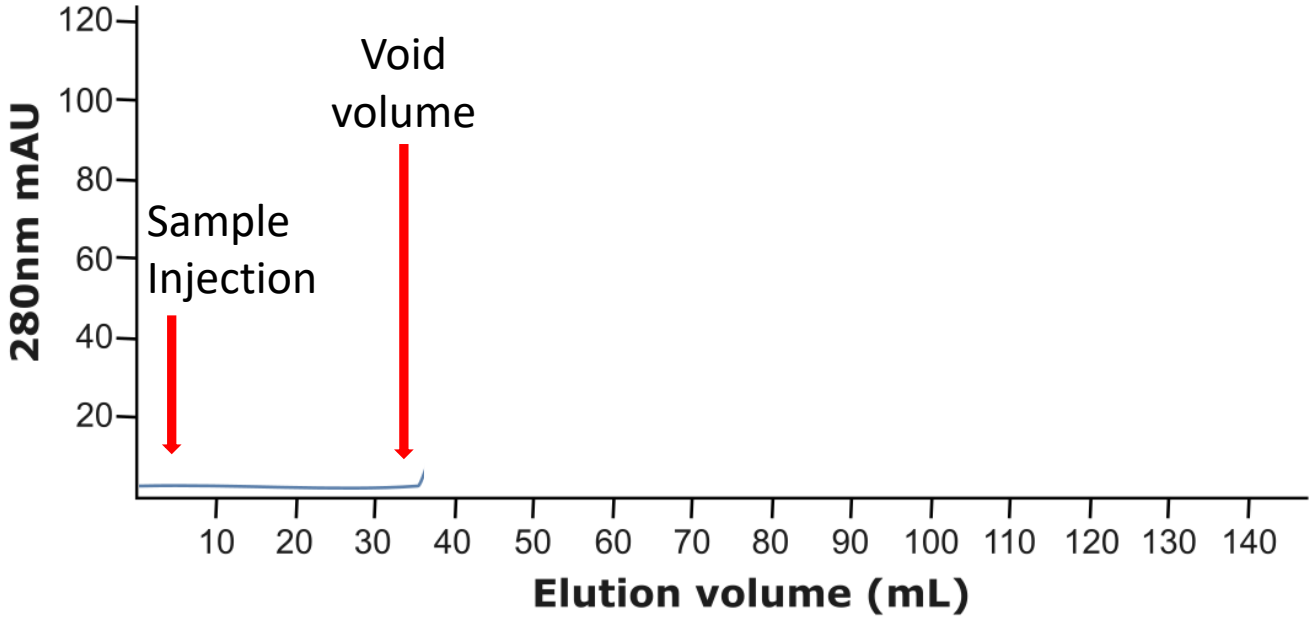
Monitoring Size Exclusion Chromatography

- The chromatogram:
  - Sample injection



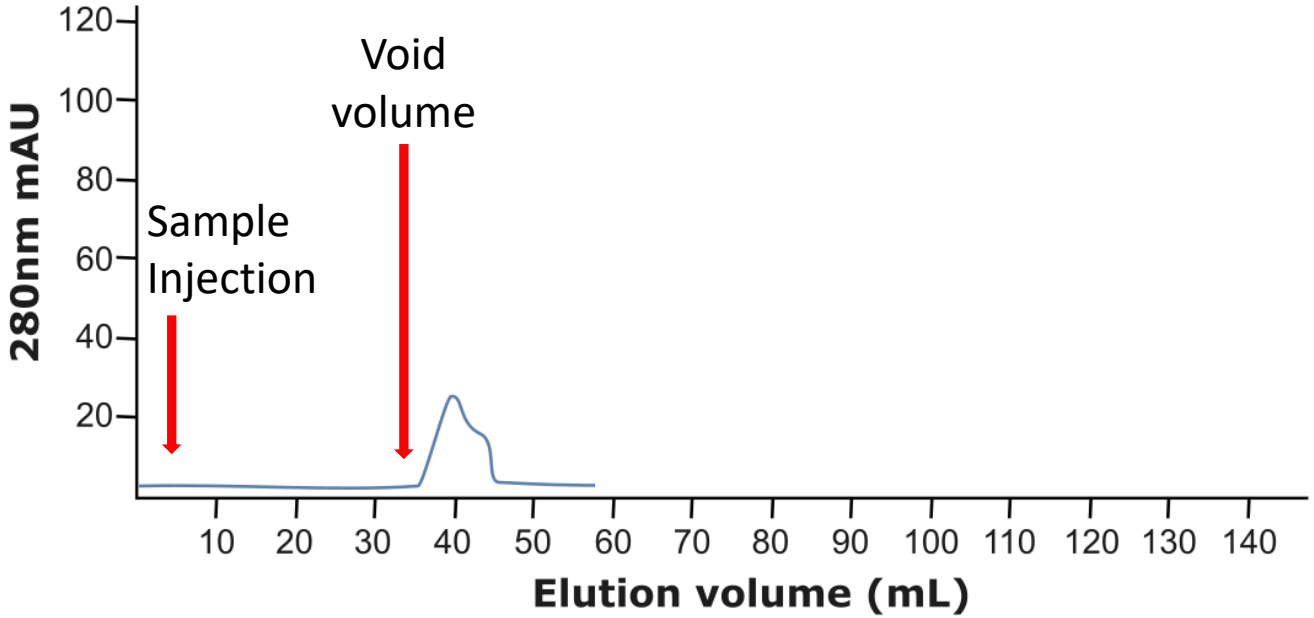
Monitoring Size Exclusion Chromatography

- The chromatogram:
  - Sample injection
  - Void volume, V_0



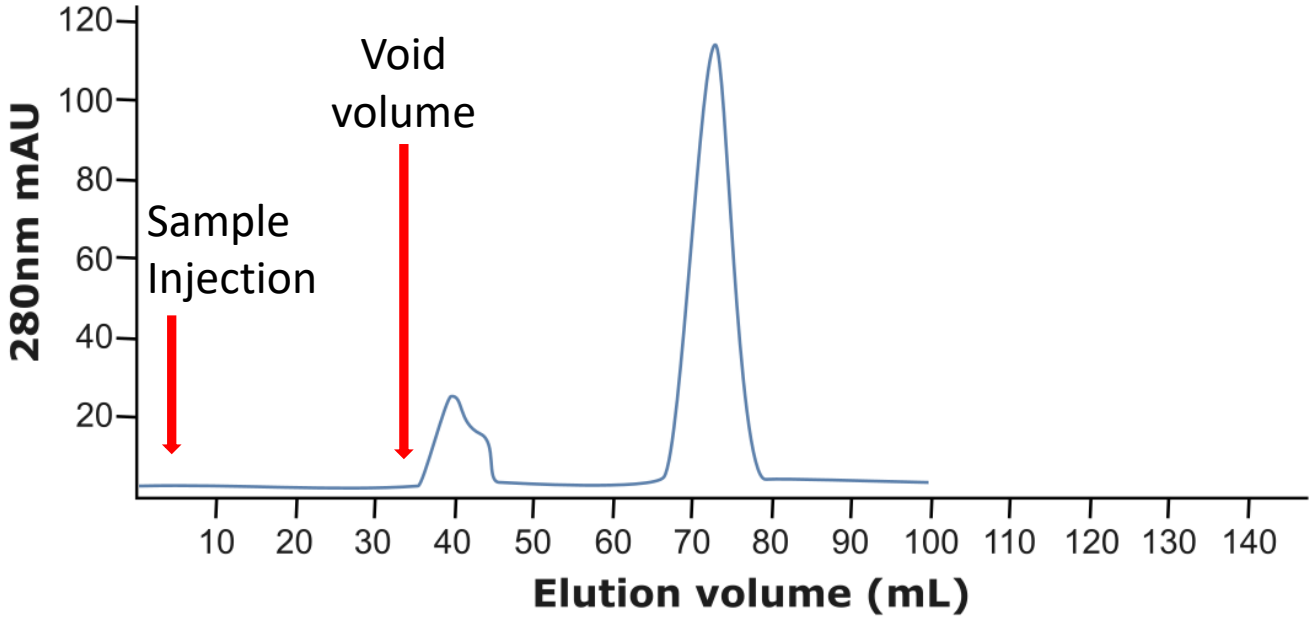
Monitoring Size Exclusion Chromatography

- The chromatogram:
  - Sample injection
  - Void volume, V_0
  - Separation range
    - Large (aggregates) first



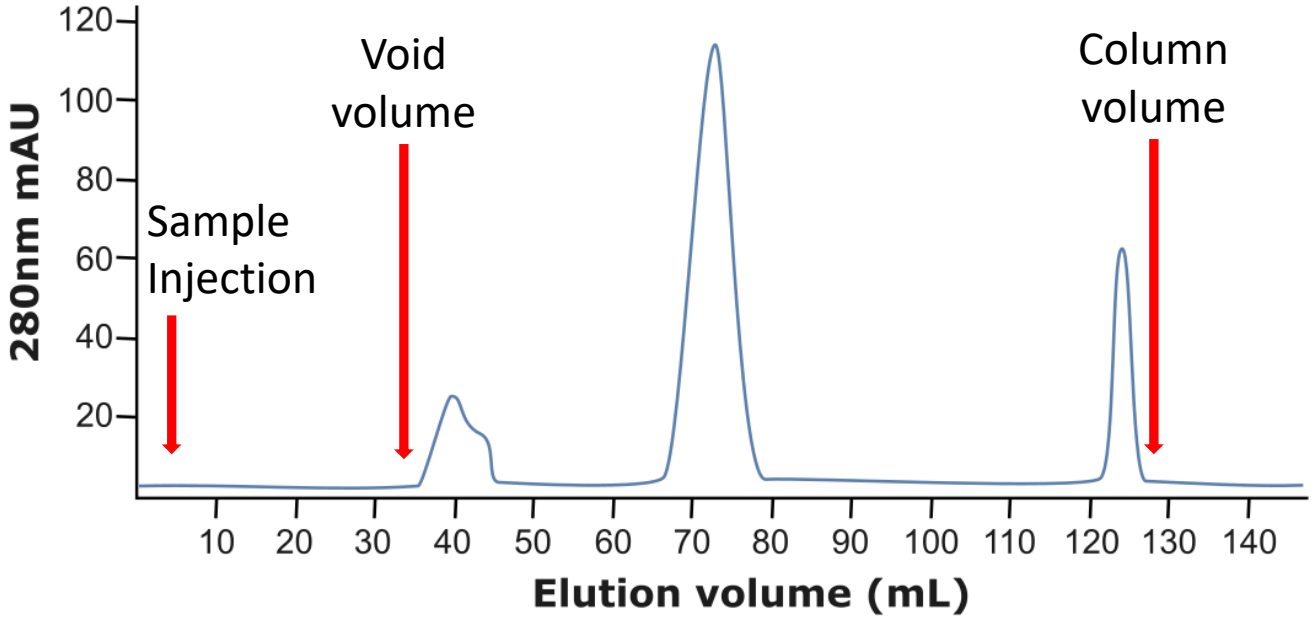
Monitoring Size Exclusion Chromatography

- The chromatogram:
  - Sample injection
  - Void volume, V_0
  - Separation range
    - Large (aggregates) first
    - Well-resolved proteins



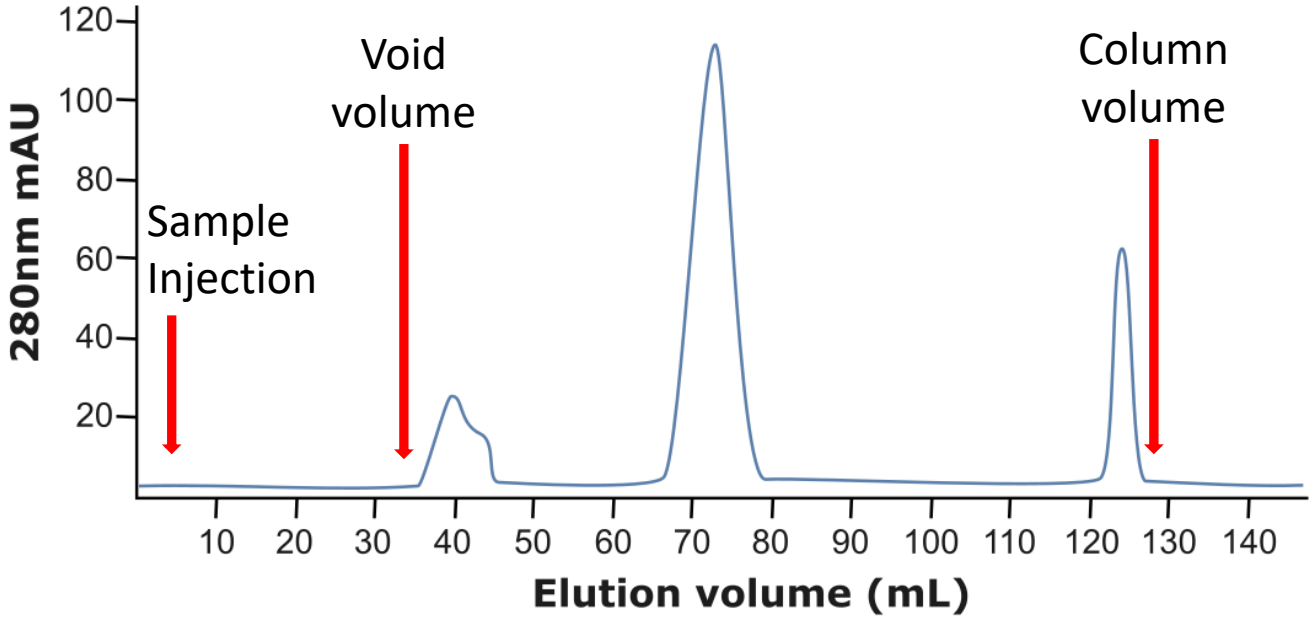
Monitoring Size Exclusion Chromatography

- The chromatogram:
  - Sample injection
  - Void volume, V_0
  - Separation range
    - Large (aggregates) first
    - Well-resolved proteins
    - Small molecules



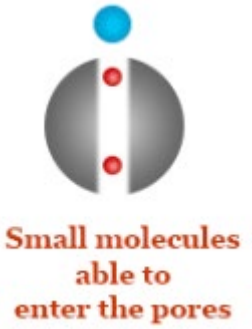
Monitoring Size Exclusion Chromatography

- The chromatogram:
  - Note this is a simple “isocratic” flow
  - One buffer, running continuously, no gradient, no elution buffer



Separation range of your column

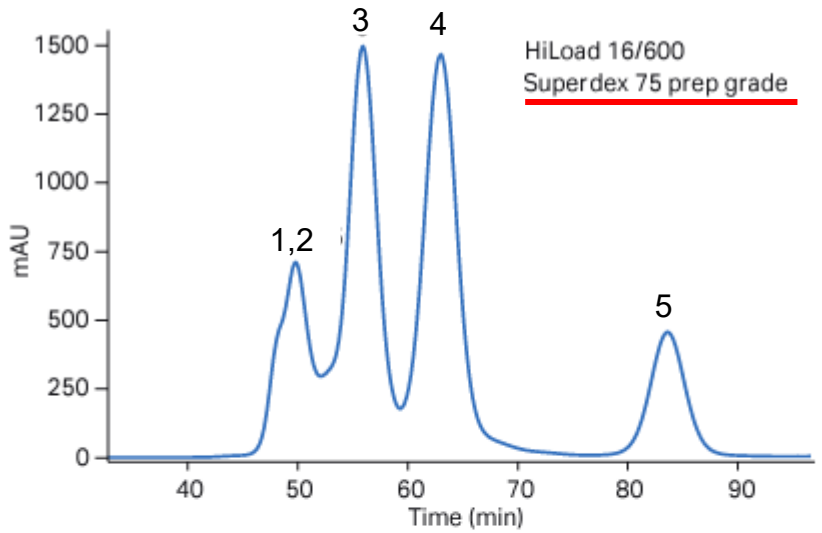
- Different pore sizes to separate different proteins

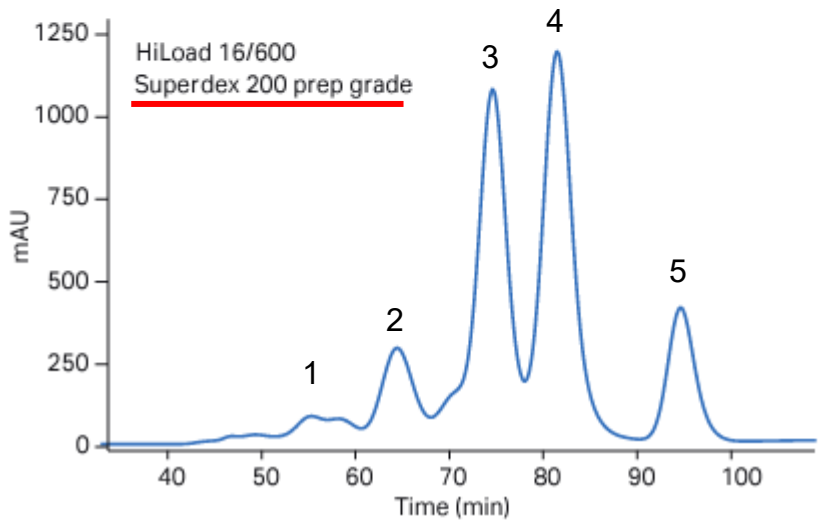


Separation range of your column

- Different pore sizes to separate different proteins

1. Ferritin	440 kDa
2. IgG	158 kDa
3. Albumin	66 kDa
4. Ovalbumin	44 kDa
5. Myoglobin	17 kDa





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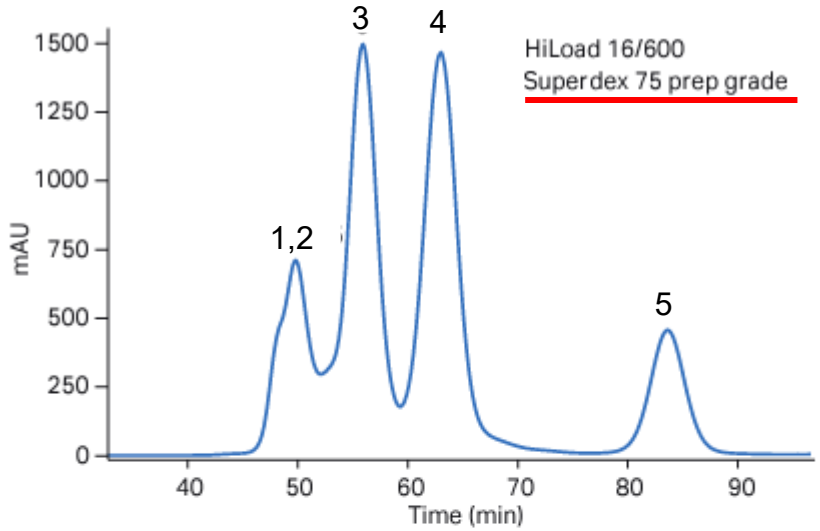
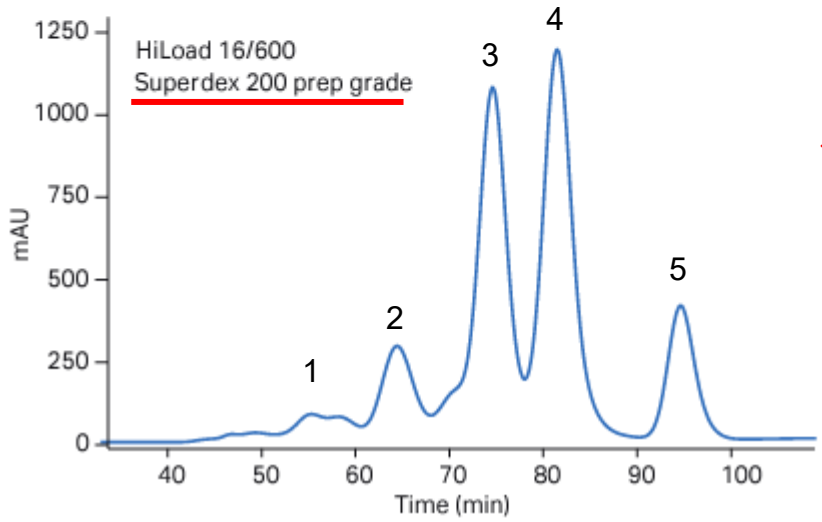


Better separation of small proteins

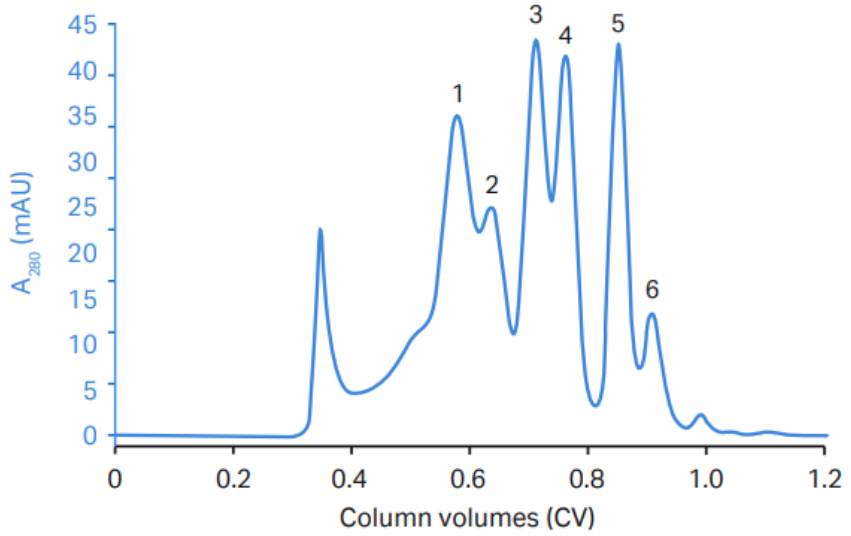


Better separation of large proteins

Separation range of your column

- For even bigger proteins
  - Superose 6 column

1. Thyroglobulin 660 kDa
2. Ferritin 440 kDa
3. Aldolase 160 kDa
4. Ovalbumin 44 kDa
5. Ribonuclease 14 kDa
6. Aprotinin 6.5 kDa



Types of Size Exclusion Chromatography

- Different column sizes for:
  - different amounts of protein
  - different resolution
  - different chromatography systems

5/150 10/300 16/600 26/600

Types of Size Exclusion Chromatography

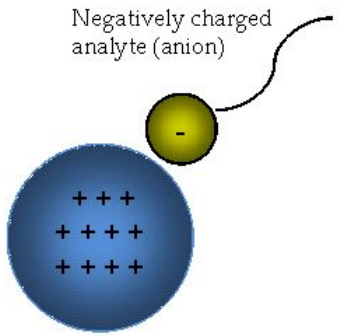
- Different column sizes for:
  - different amounts of protein
  - different resolution
  - different chromatography systems

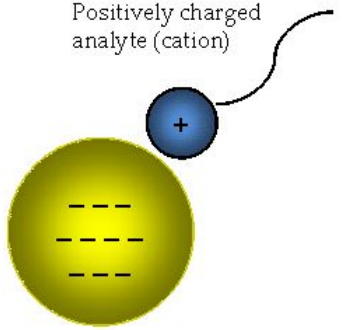
What if size doesn't separate my contaminants?

- Although SEC is excellent for removing large aggregates and exchanging small buffer components…
- …sometimes the contaminants in our samples are the same size
- So we use different chromatography techniques to separate these

Ion Exchange Chromatography

- Separates based on charge





Ion Exchange Chromatography

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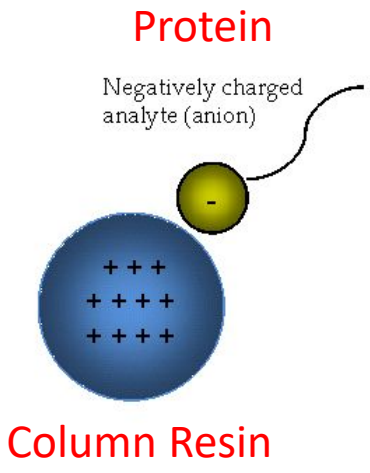
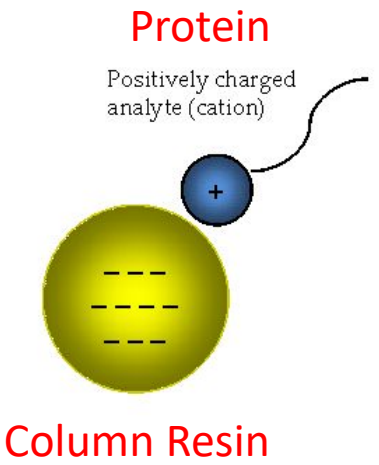
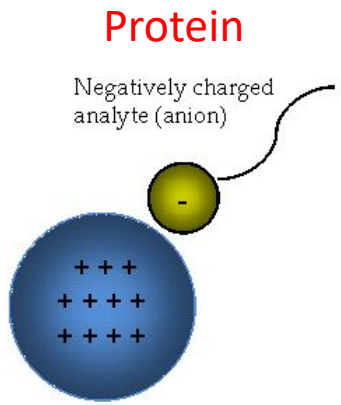




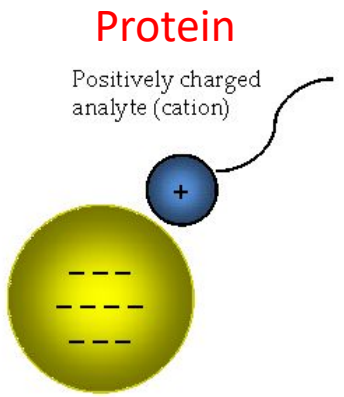
Ion Exchange Chromatography

- Separates based on charge
- Anion and cation exchange



Column Resin

Anion Exchange

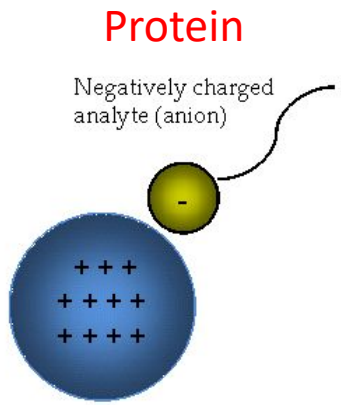


Column Resin

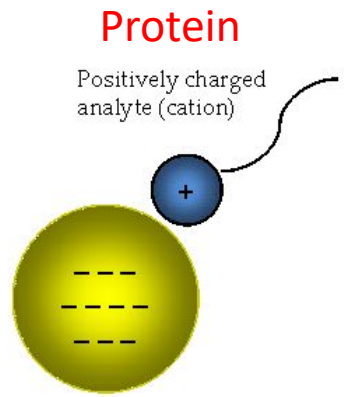
Cation Exchange

Ion Exchange Chromatography

- Separates based on charge
- Anion and cation exchange
- How do I know what charge my protein is?



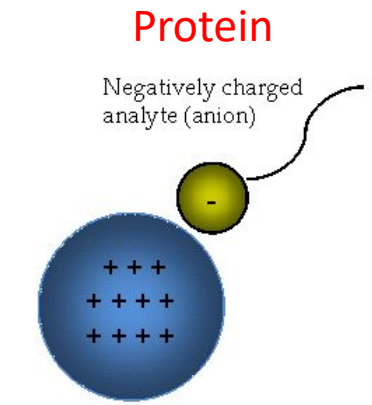
Anion Exchange



Cation Exchange

Ion Exchange Chromatography

- Separates based on charge
- Anion and cation exchange
- How do I know what charge my protein is?
- Using ProtParam as described on Day 2



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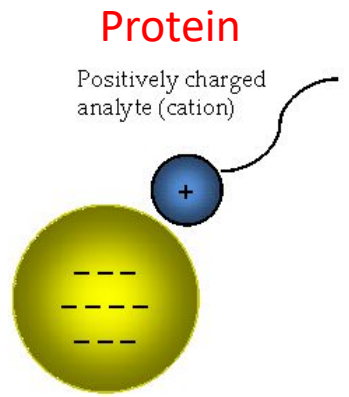


Cation Exchange

My protein charge

- Protparam calculates a theoretical pI
- This is the pH where your protein is uncharged

Number of amino acids: 510

Molecular weight: 57364.86

Theoretical pI: 6.37

Amino acid composition:

Ala (A)	31	6.1%
Arg (R)	34	6.7%
Asn (N)	30	5.9%
Asp (D)	35	6.9%
Cys (C)	9	1.8%
Gln (Q)	24	4.7%
Glu (E)	27	5.3%
Gly (G)	32	6.3%
His (H)	9	1.8%
Ile (I)	23	4.5%
Leu (L)	45	8.8%
Lys (K)	25	4.9%
Met (M)	11	2.2%
Phe (F)	16	3.1%
Pro (P)	34	6.7%
Ser (S)	32	6.3%
Thr (T)	33	6.5%
Trp (W)	7	1.4%
Tyr (Y)	18	3.5%
Val (V)	35	6.9%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%

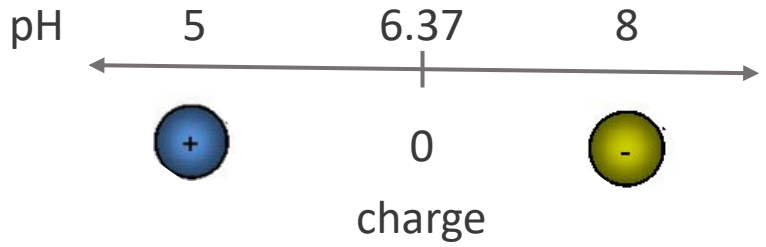
My protein charge

- Protparam calculates a theoretical pI
- This is the pH where your protein is uncharged
- Above this it is –ve, below it is +ve

Number of amino acids: 510

Molecular weight: 57364.86

Theoretical pI: 6.37



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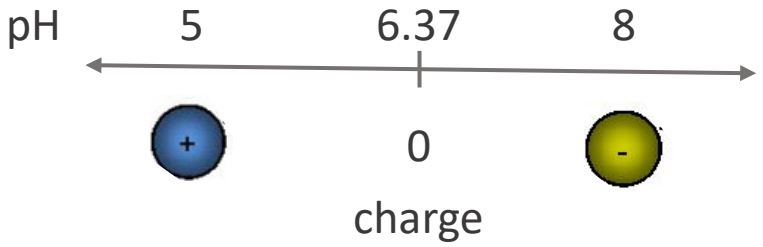
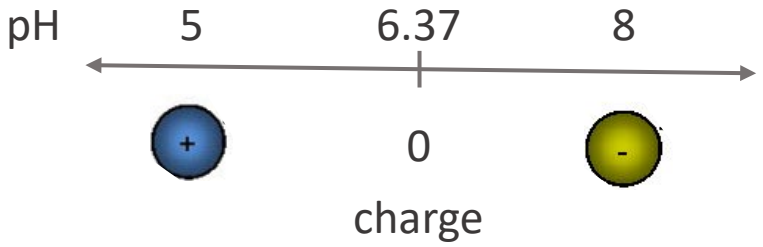


Ion exchange chromatography

- For this protein we could do:
  - Cation exchange in buffer @ pH 5

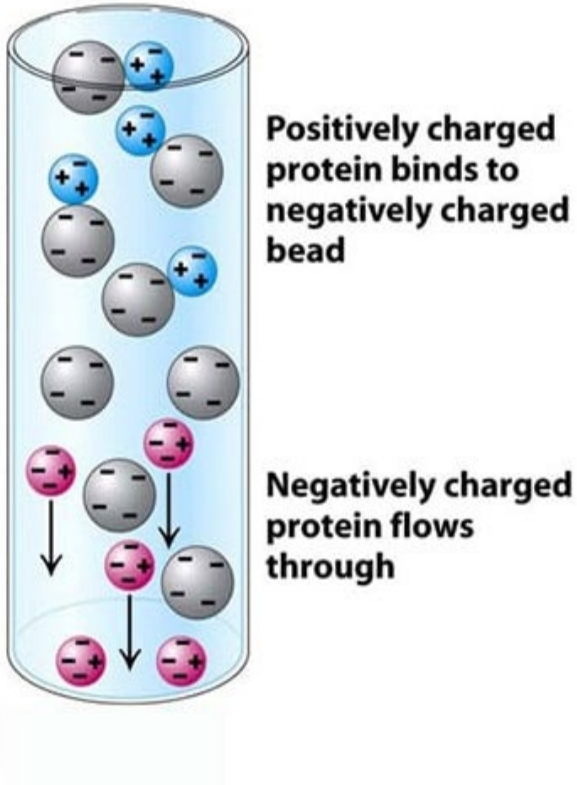
  OR

  - Anion exchange in buffer @pH 8



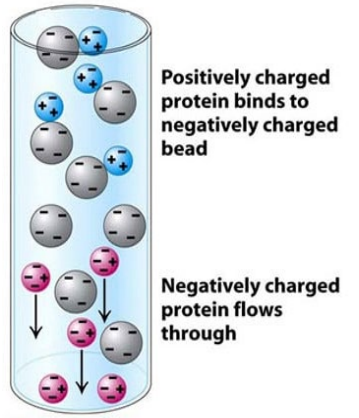
Cation Exchange Chromatography

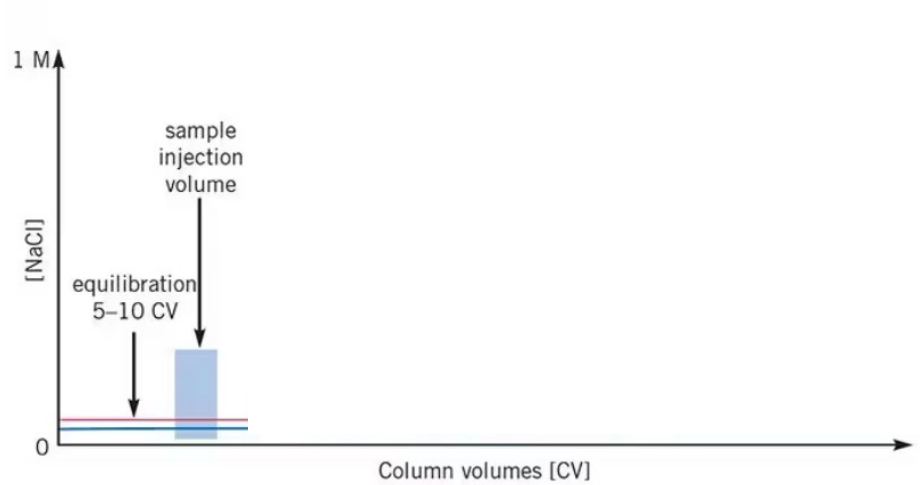
- Using cation exchange:
  - positively charged protein binds the column
  - negatively charged protein flows through



Cation Exchange Chromatography

- Using cation exchange:
  - positively charged protein binds the column
  - negatively charged protein flows through
  - Load the column in low [salt]





Cation Exchange Chromatography

- Using cation exchange:
  - positively charged protein binds the column
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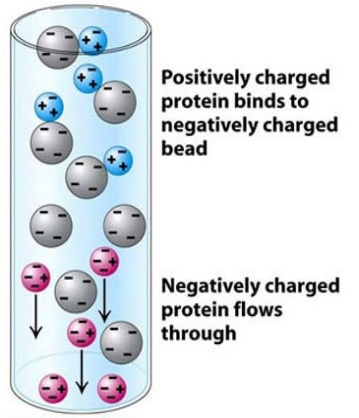
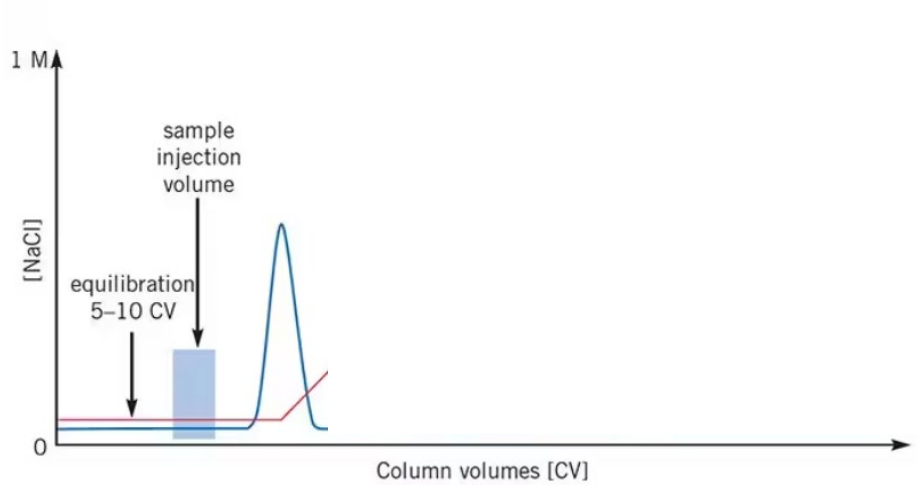
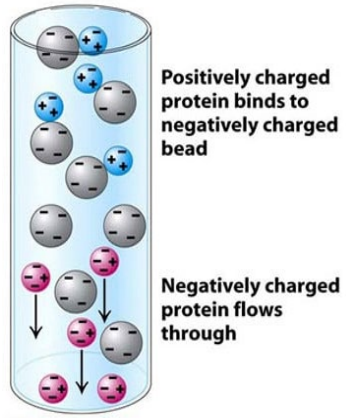
  - Load the column in low [salt]
  - Negative proteins don’t bind

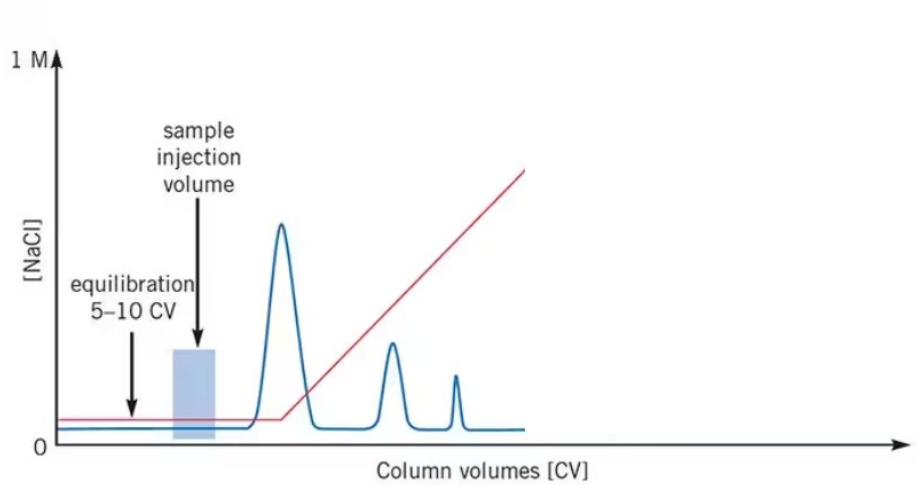




Cation Exchange Chromatography

- Using cation exchange:
  - positively charged protein binds the column
  - negatively charged protein flows through
  - Load the column in low [salt]
  - Negative proteins don’t bind
  - Elute using increasing [salt]





Cation Exchange Chromatography

- Using cation exchange:
  - positively charged protein binds the column
  - negatively charged protein flows through
  - Load the column in low [salt]
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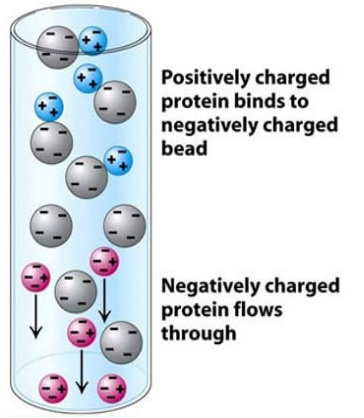
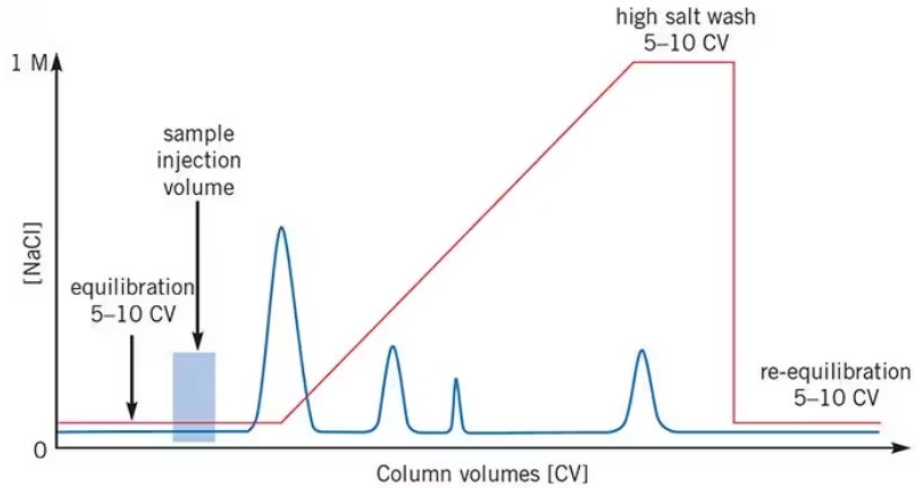
  - Elute using increasing [salt]
  - Wash with high [salt]



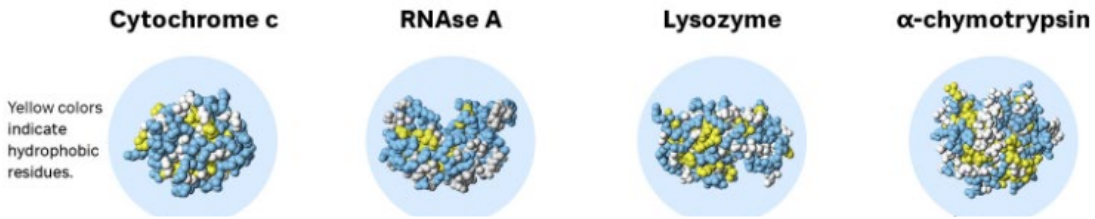


Other chromatography techniques

- Hydrophobic interaction chromatography

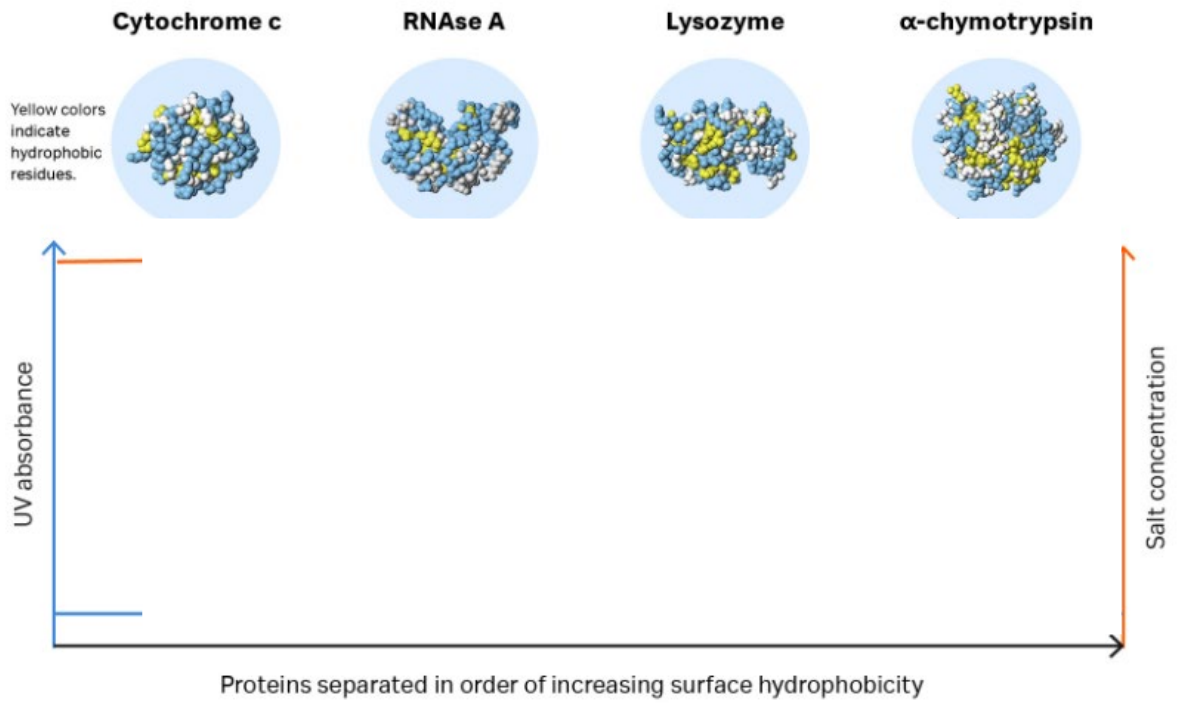
Other chromatography techniques

- Hydrophobic interaction chromatography
  - Separates based on hydrophobicity



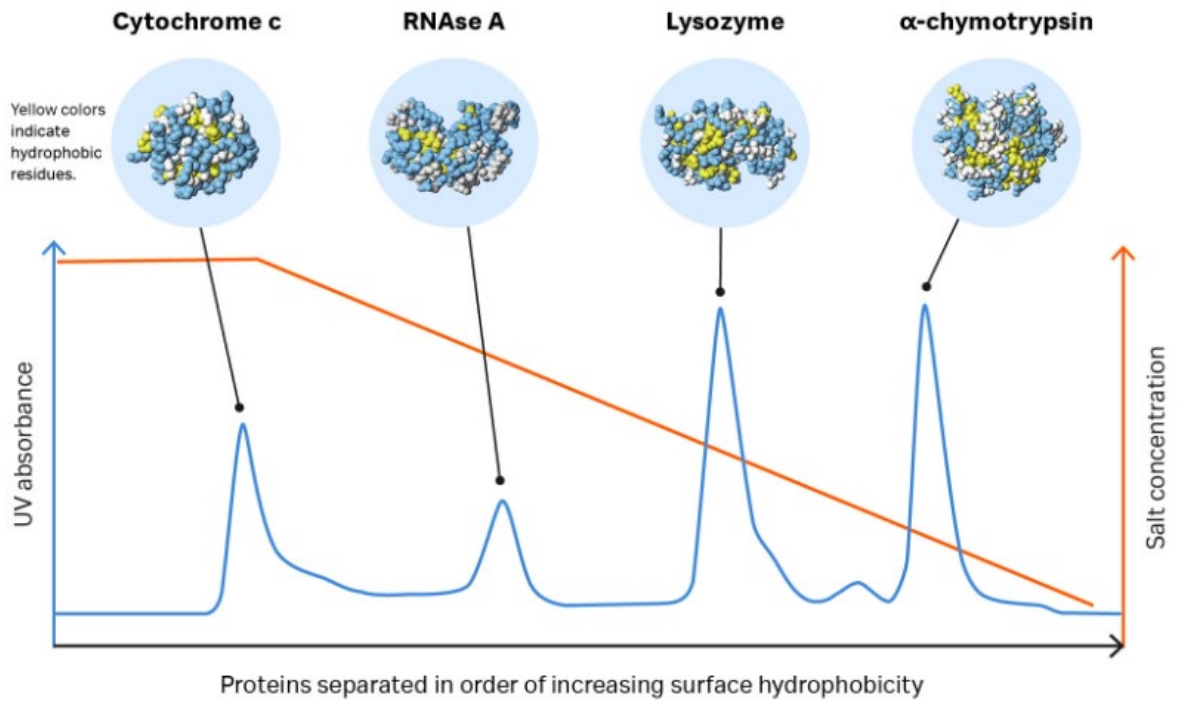
Other chromatography techniques

- Hydrophobic interaction chromatography
  - Separates based on hydrophobicity
  - Elute using salt but this time
  - Start in high [salt]



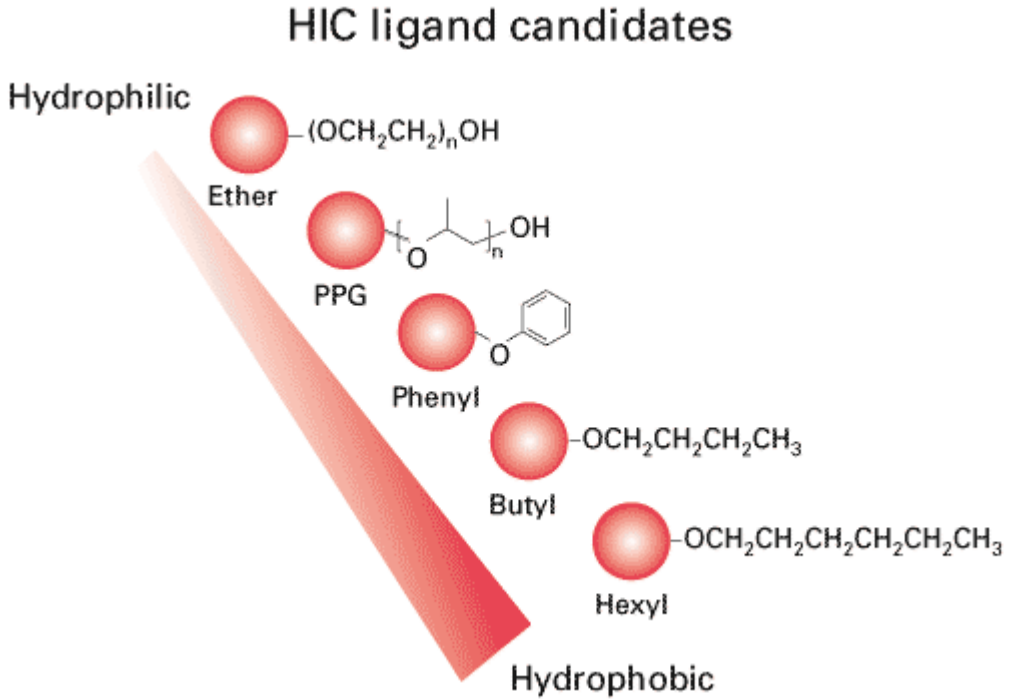
Other chromatography techniques

- Hydrophobic interaction chromatography
  - Separates based on hydrophobicity
  - Elute using salt but this time
  - Start in high [salt]
  - Separate with decreasing [salt]



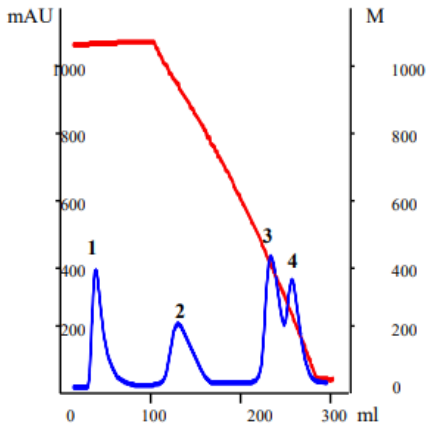
Other chromatography techniques

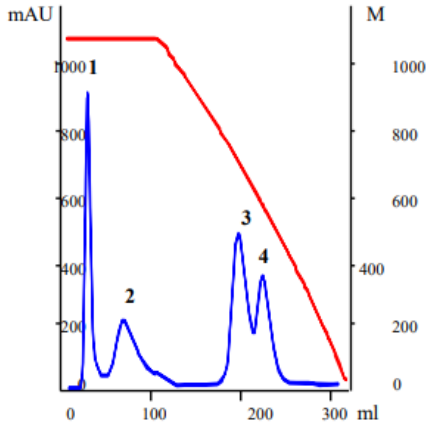
- Hydrophobic interaction chromatography
  - Choice of resins

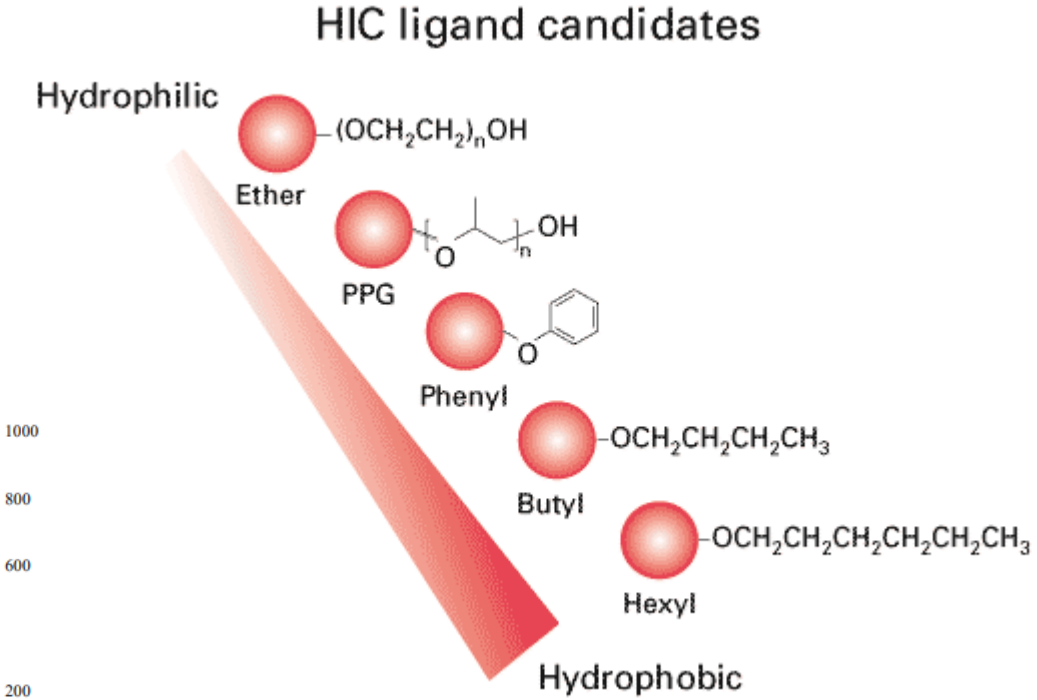


Other chromatography techniques

- Hydrophobic interaction chromatography
  - Choice of resins
  - Increasing hydrophobicity for different separation







Chromatography Equipment

- Manual equipment – gravity columns
- Simple liquid handling - peristaltic pumps
- Automated systems – high performance liquid chromatography
  - Range of instruments from simple to complex
  - Range of suppliers
- All do essentially the same thing, pump liquid through a column

Manual equipment – gravity columns

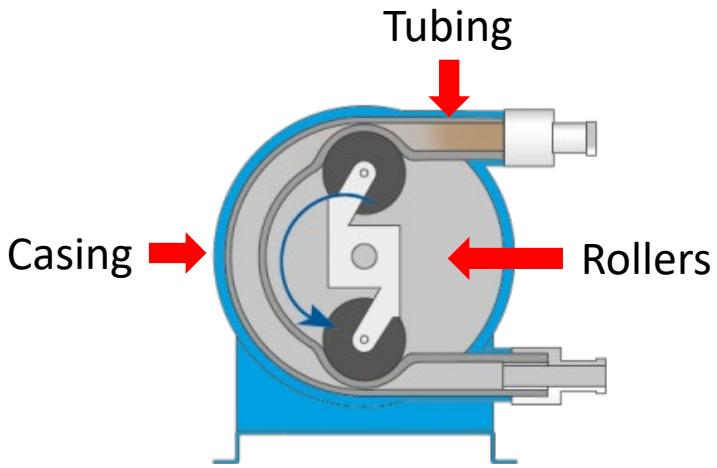
- Best when using loose resin
- Manually load resin into columns
- Can run via gravity alone
  - You used these yesterday

Manual equipment – gravity columns

- Best when using loose resin
- Manually load resin into columns
- Can run via gravity alone
  - You used these yesterday
- Come in a range of sizes depending on the size of your prep and how much protein you have

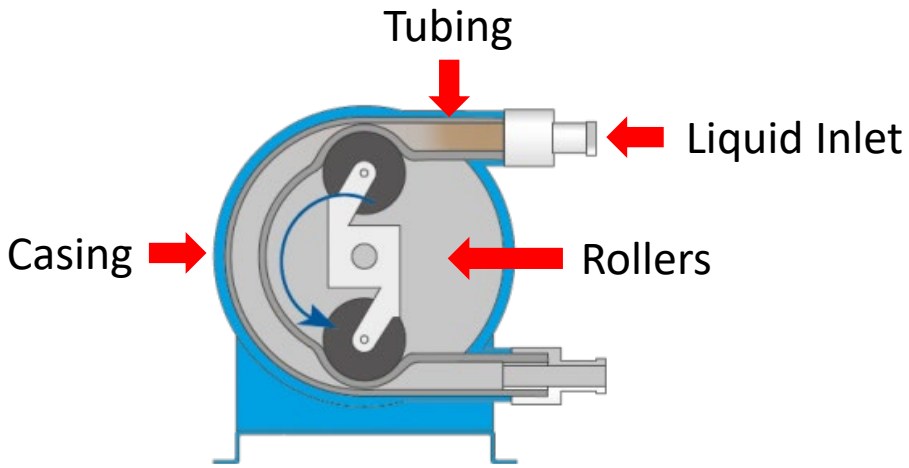
Simple liquid handling – peristaltic pumps

- The theory



Simple liquid handling – peristaltic pumps

- The theory



Simple liquid handling – peristaltic pumps

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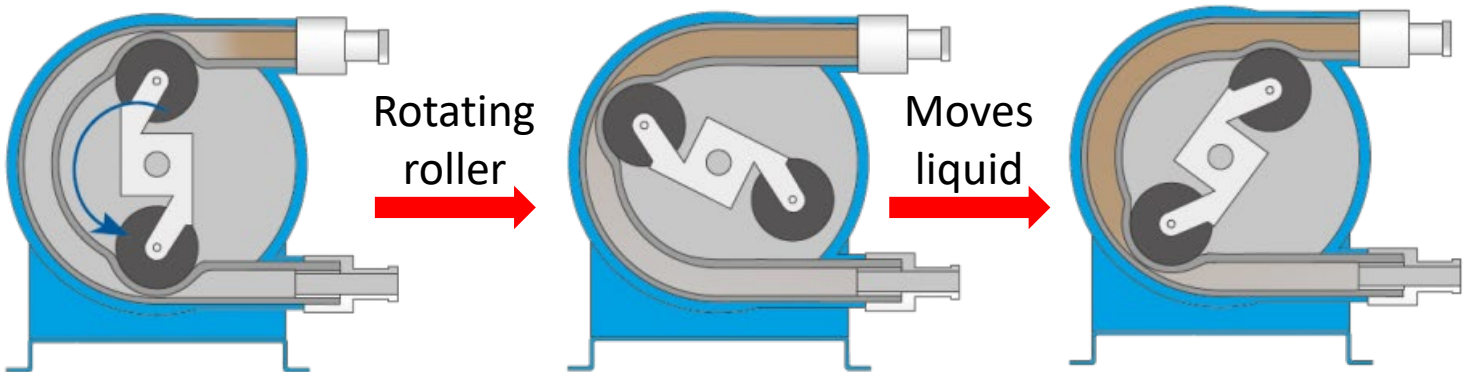
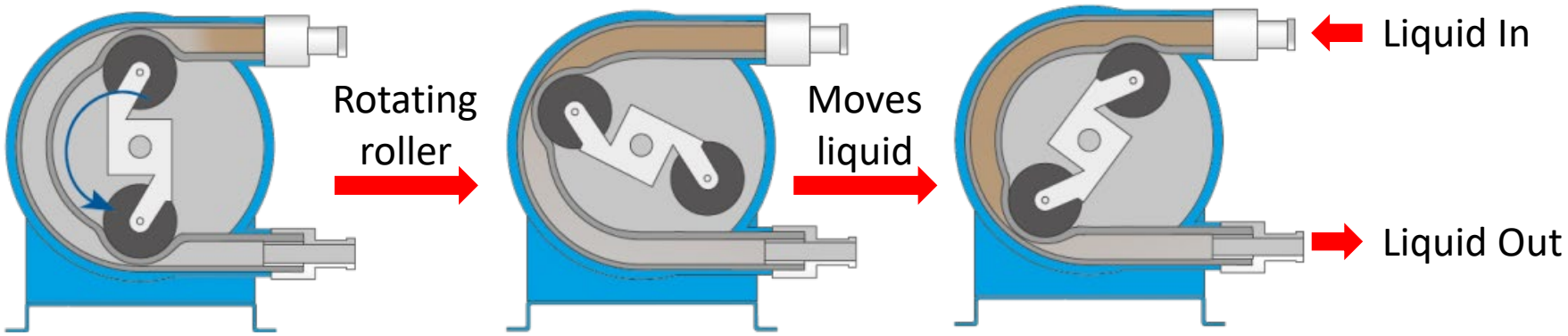


Simple liquid handling – peristaltic pumps

- The theory



Simple liquid handling – peristaltic pumps

- Come in a range of styles/models

Liquid In

Liquid Out

Liquid In

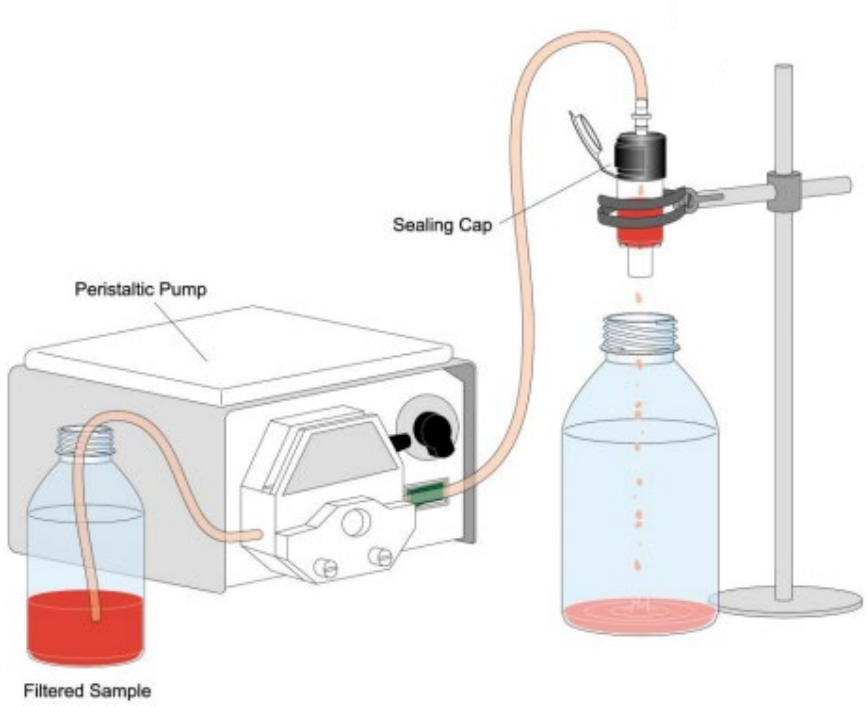
Liquid Out

Liquid In

Liquid Out

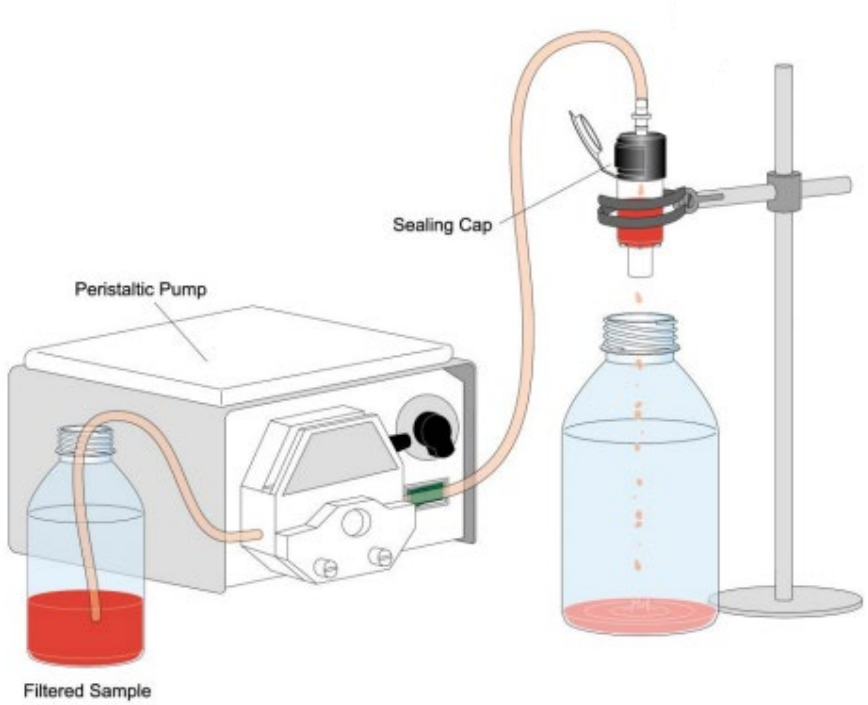
Peristaltic pump for chromatography

- Can connect peristaltic pumps to:
  - gravity columns
  - small pre-packed columns



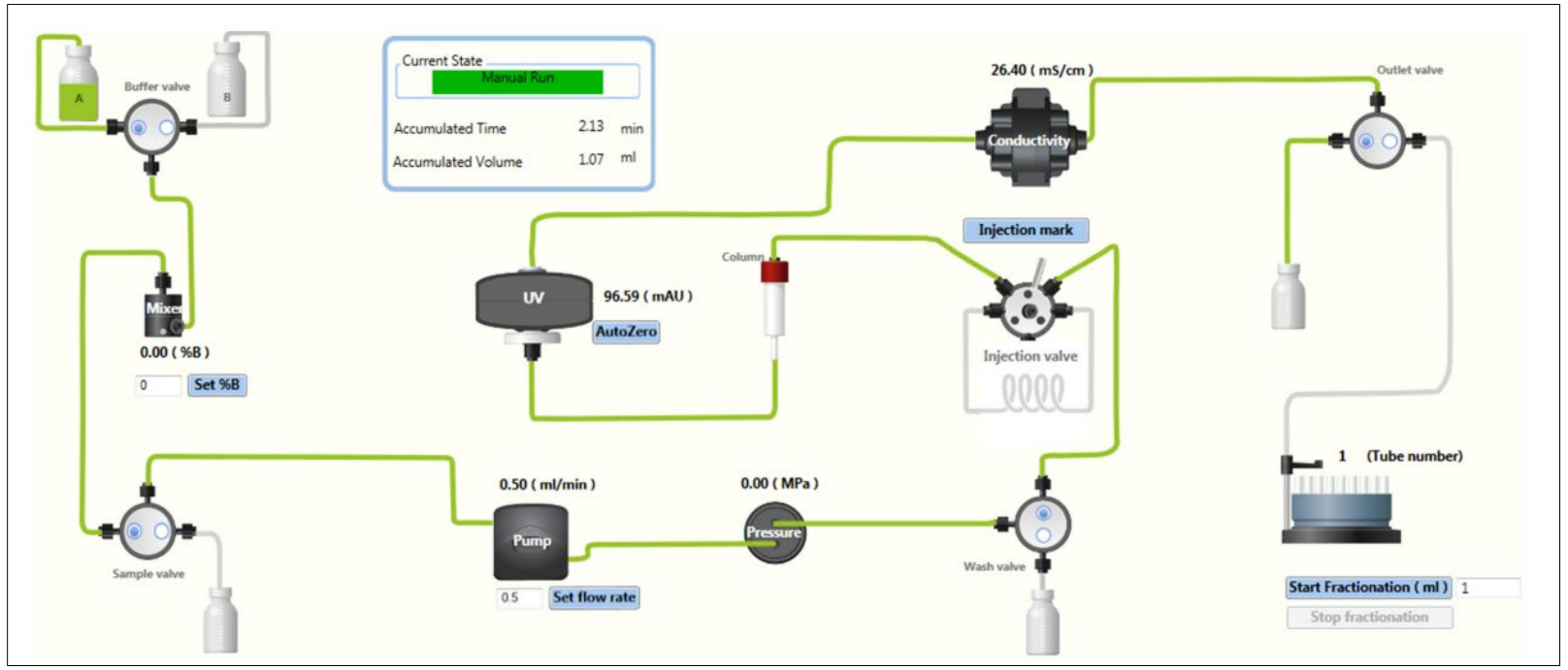
Peristaltic pump for chromatography

- Can connect peristaltic pumps to:
  - gravity columns
  - small pre-packed columns
- Need to:
  - manually collect elution
  - ensure sample/buffer doesn't run out



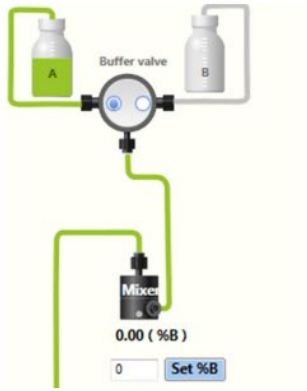
Automated systems – AKTA Start

- Components



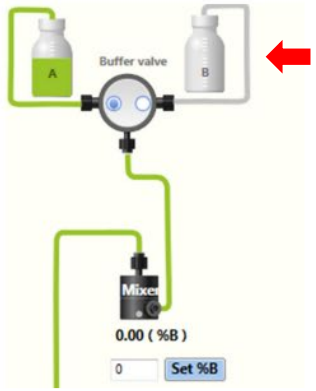
Automated systems – AKTA Start

- Components



Automated systems – AKTA Start

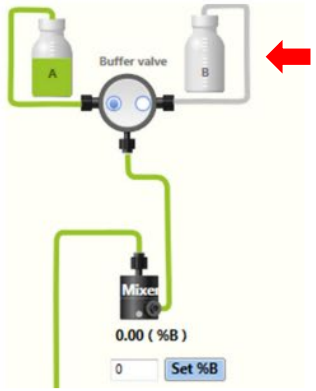
- Components



Two Inlets
Allow use of gradients needed for ion exchange and HIC

Automated systems – AKTA Start

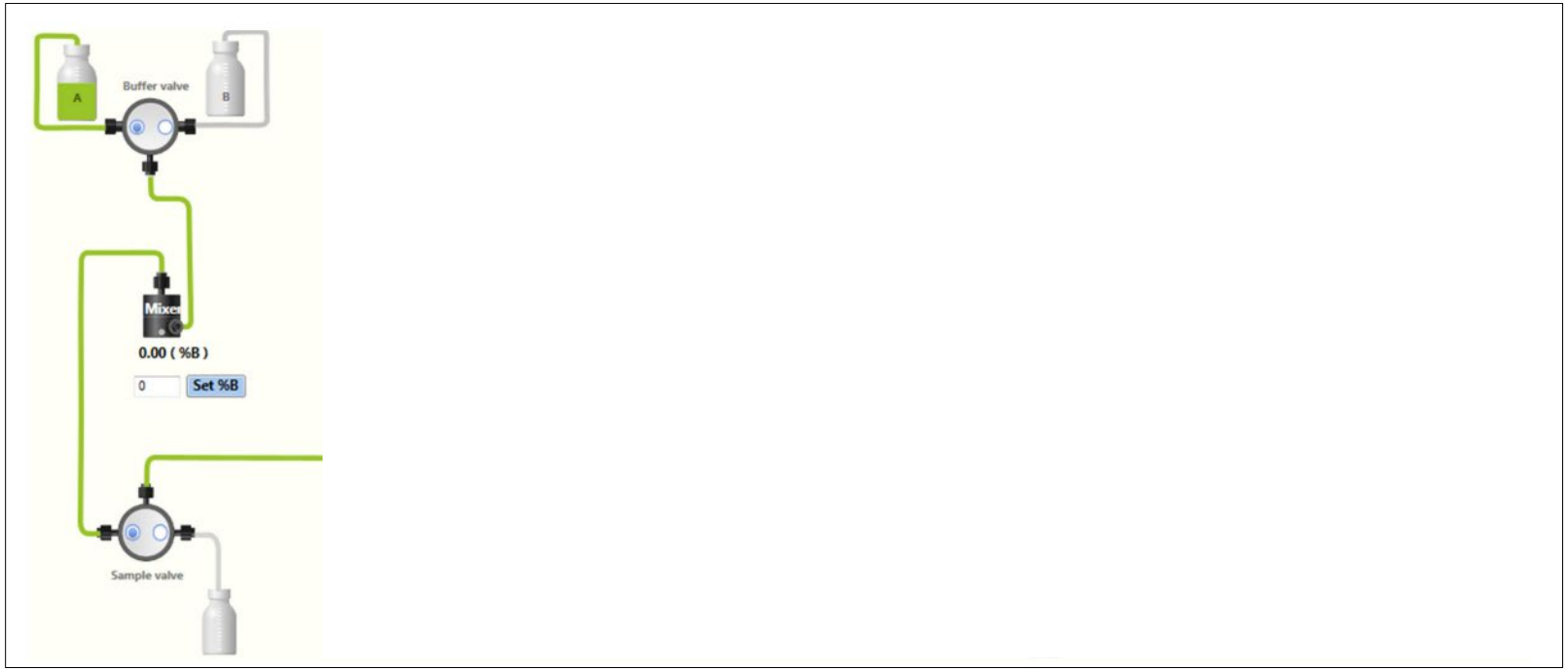
- Components

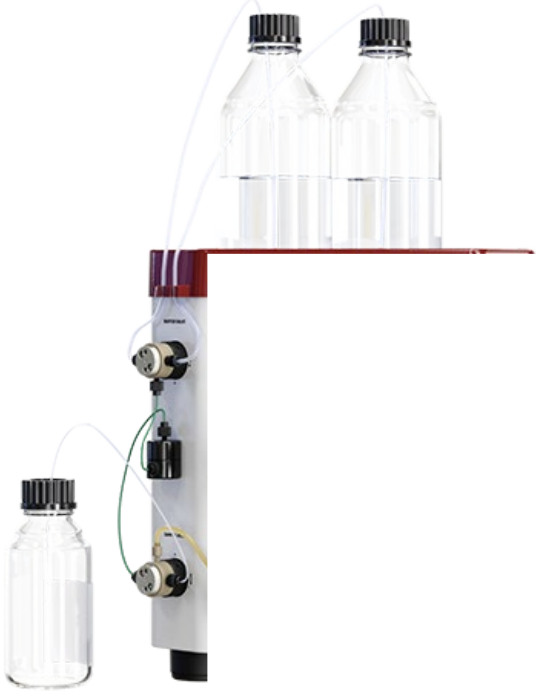


Two Inlets
Allow use of gradients needed for ion exchange and HIC
For SEC we only need one buffer (A)

Automated systems – AKTA Start

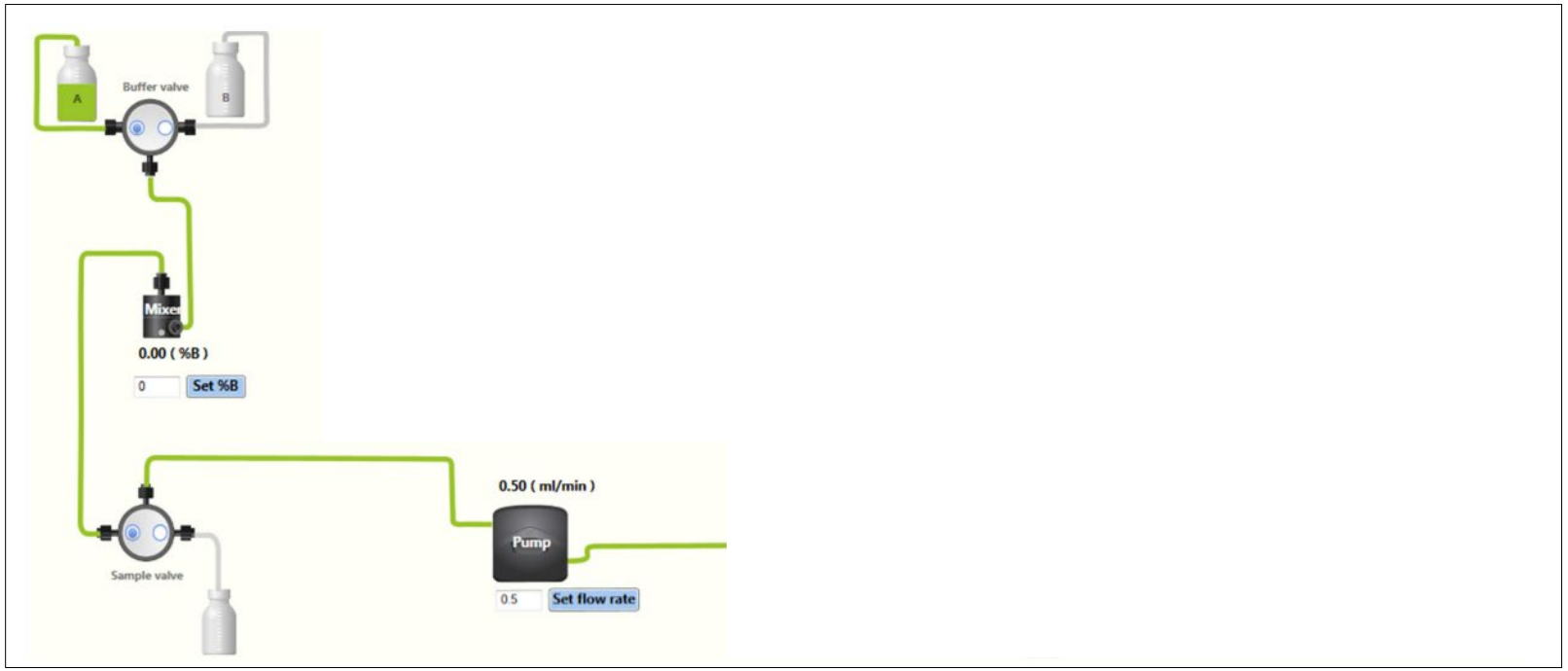
- Components

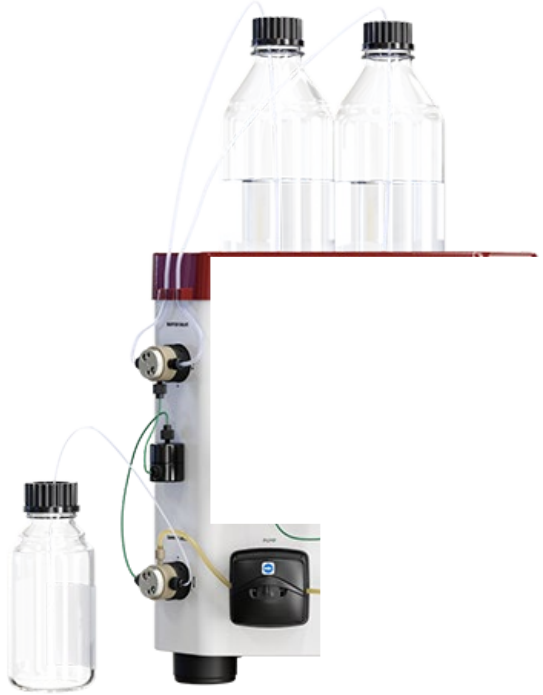




Automated systems – AKTA Start

- Components





Automated systems – AKTA Start

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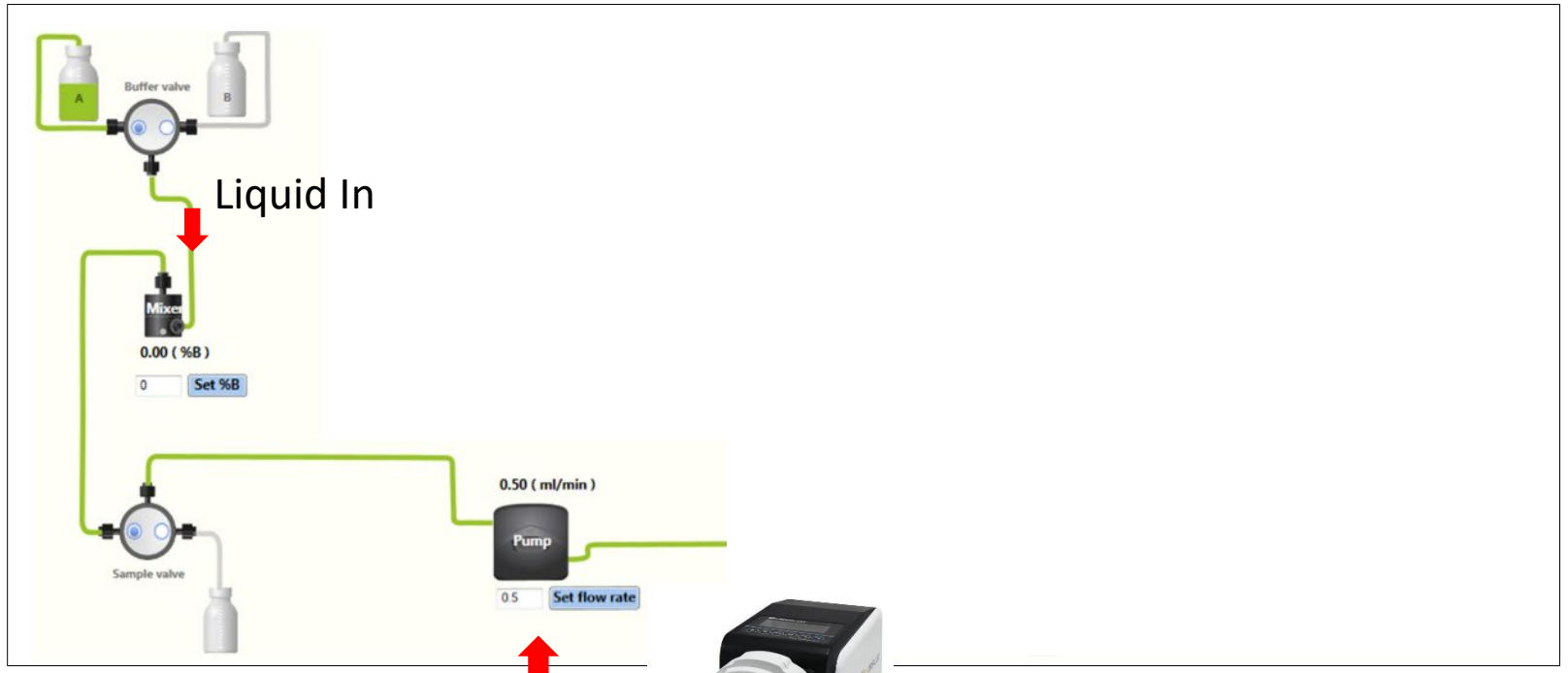
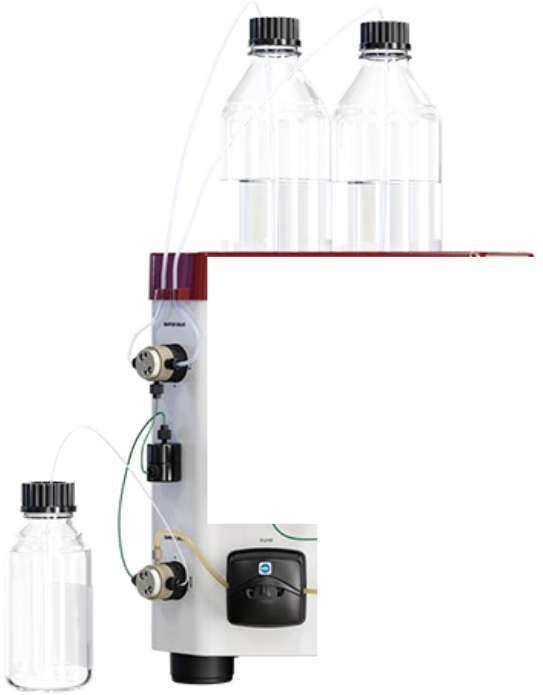
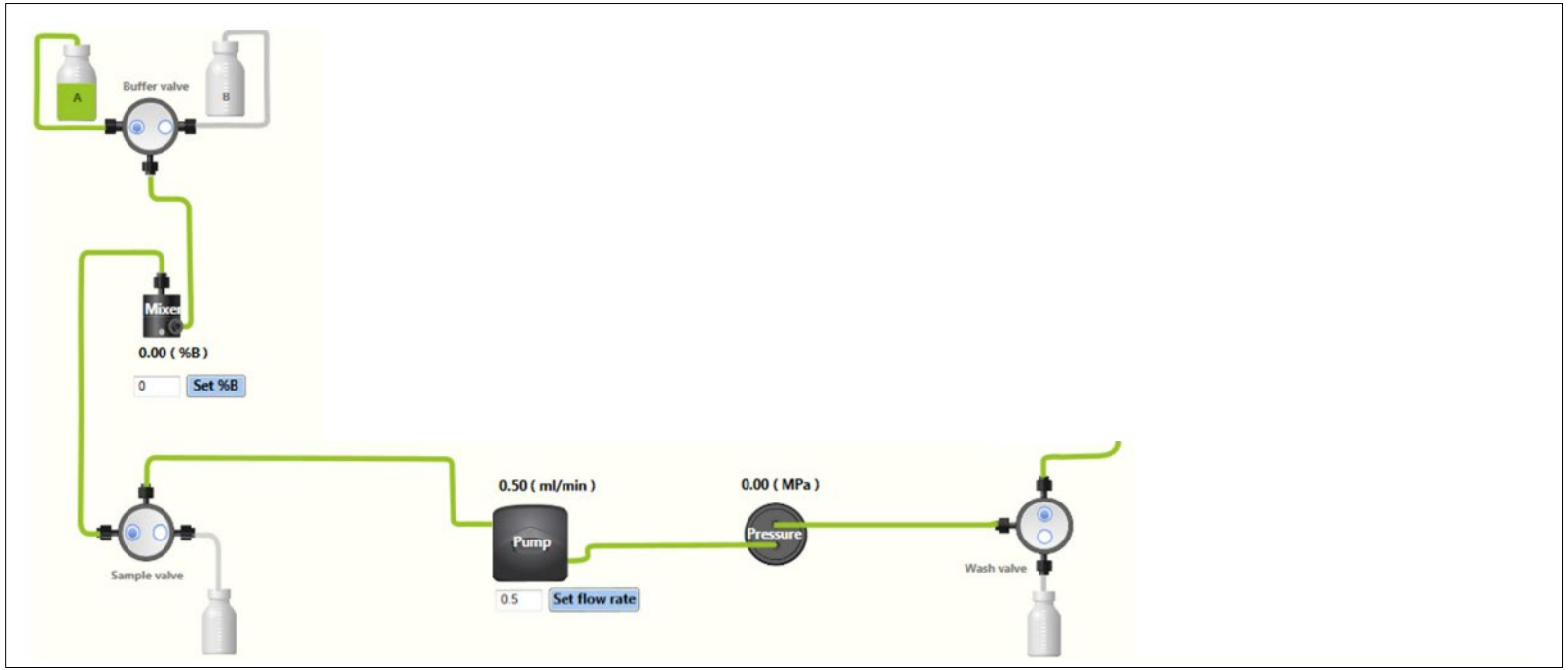


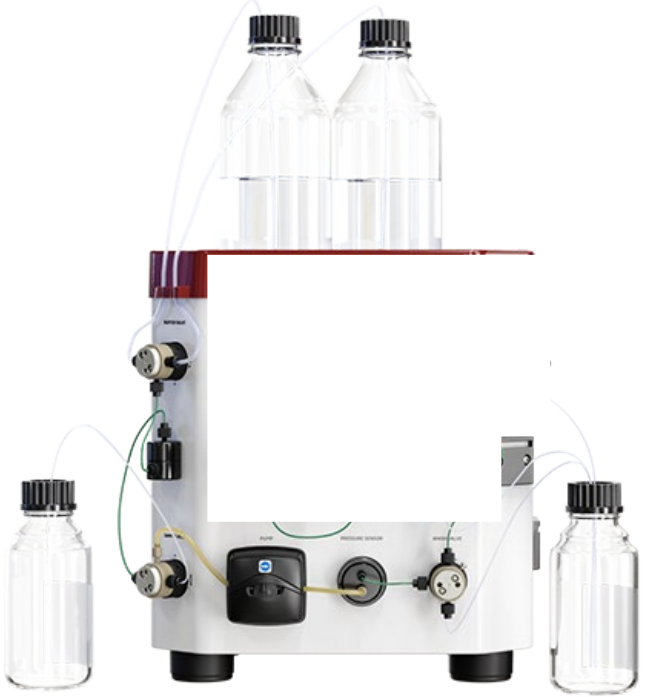
Peristaltic pump



Automated systems – AKTA Start

- Components





Automated systems – AKTA Start

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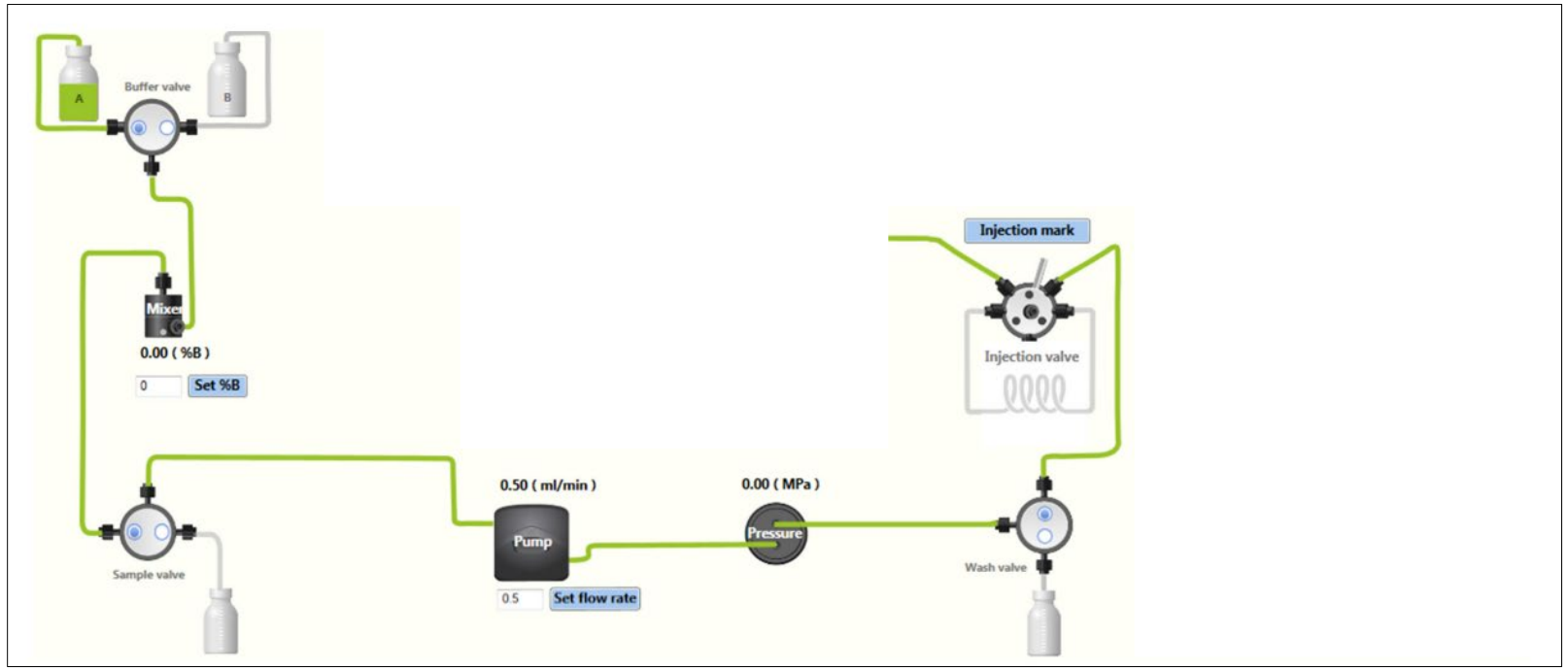

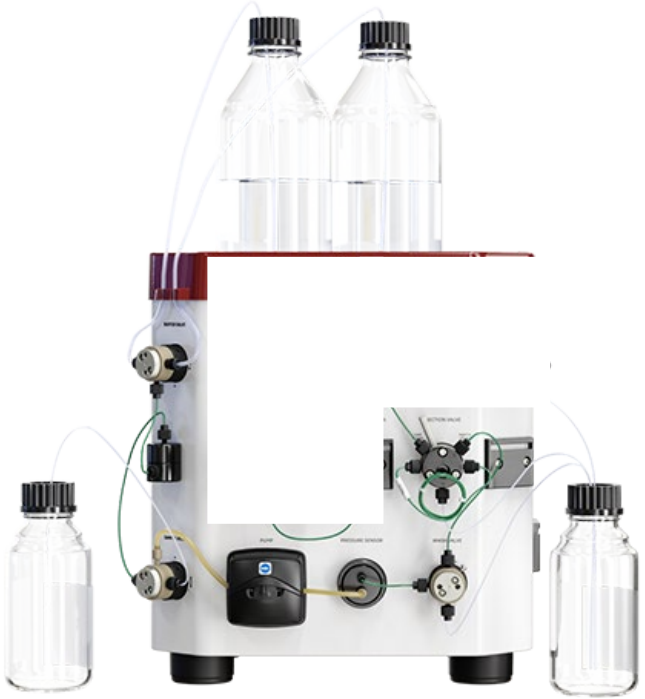

Automated systems – AKTA Start

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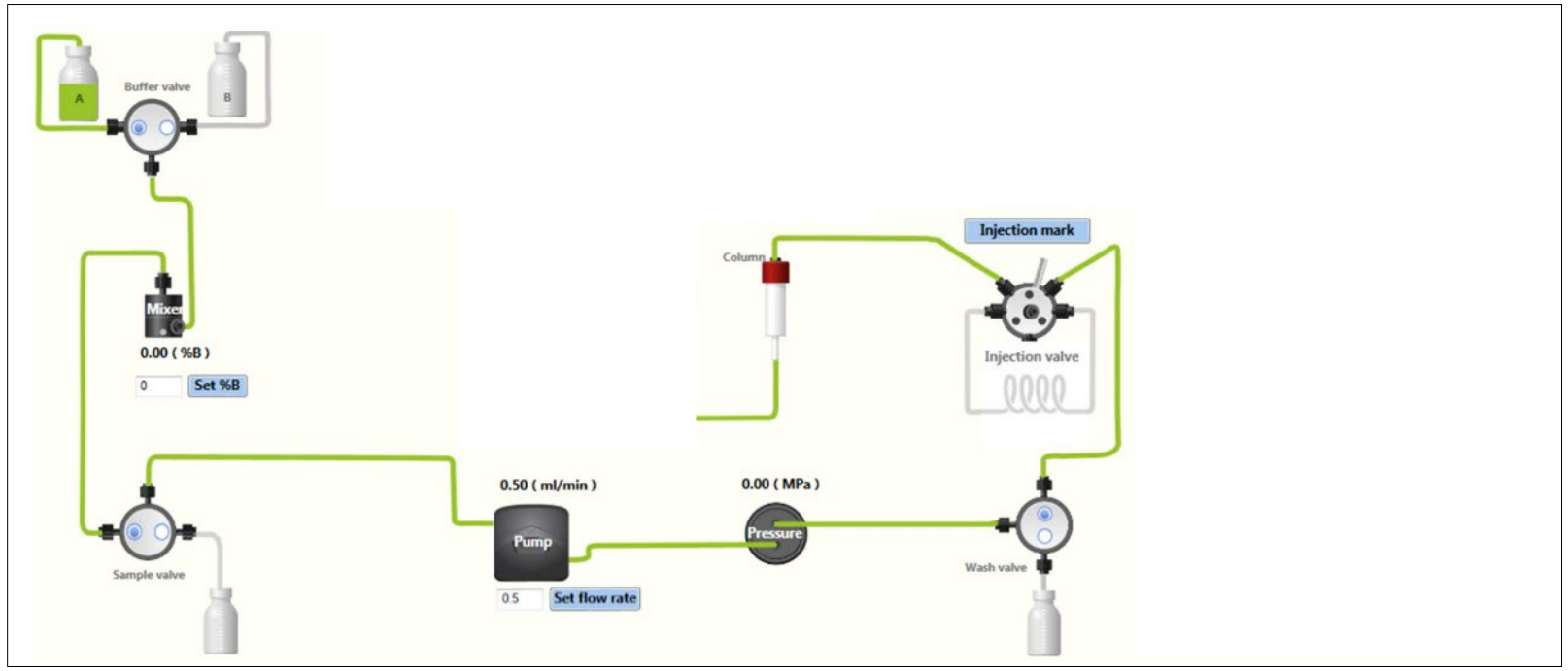

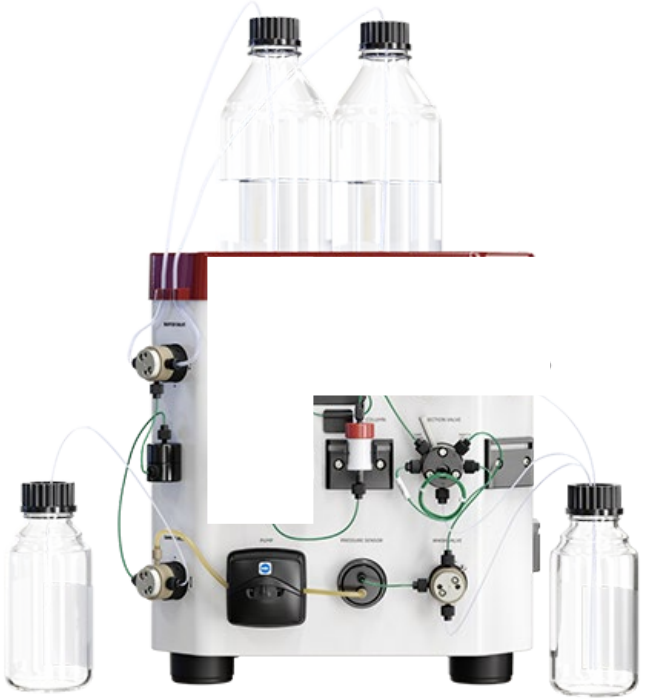

Automated systems – AKTA Start

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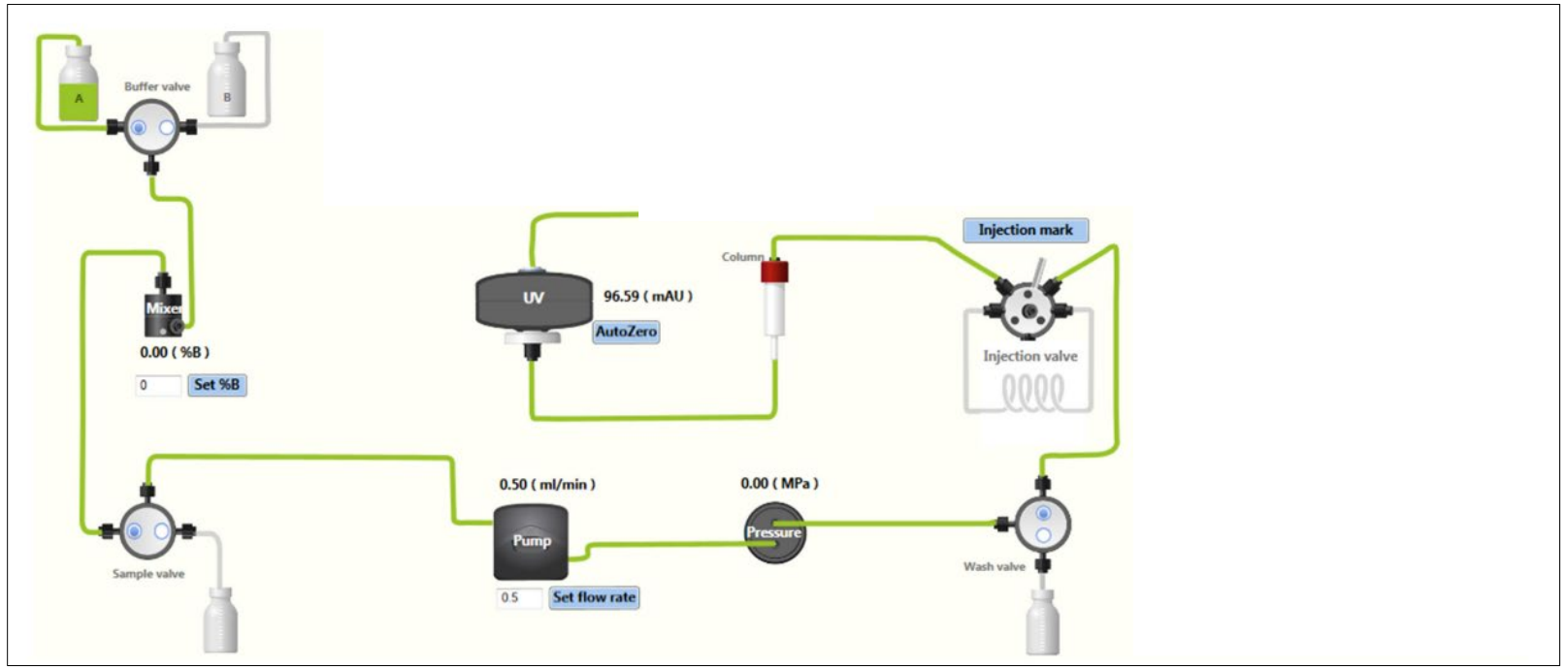
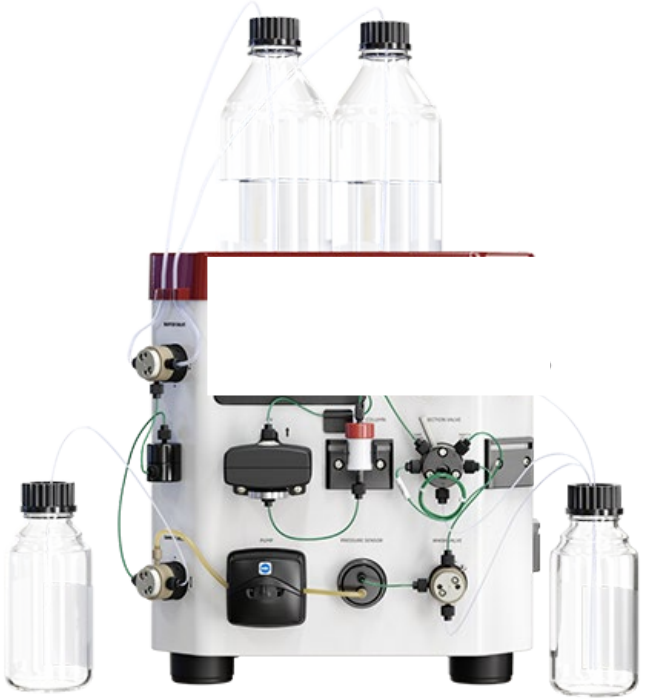
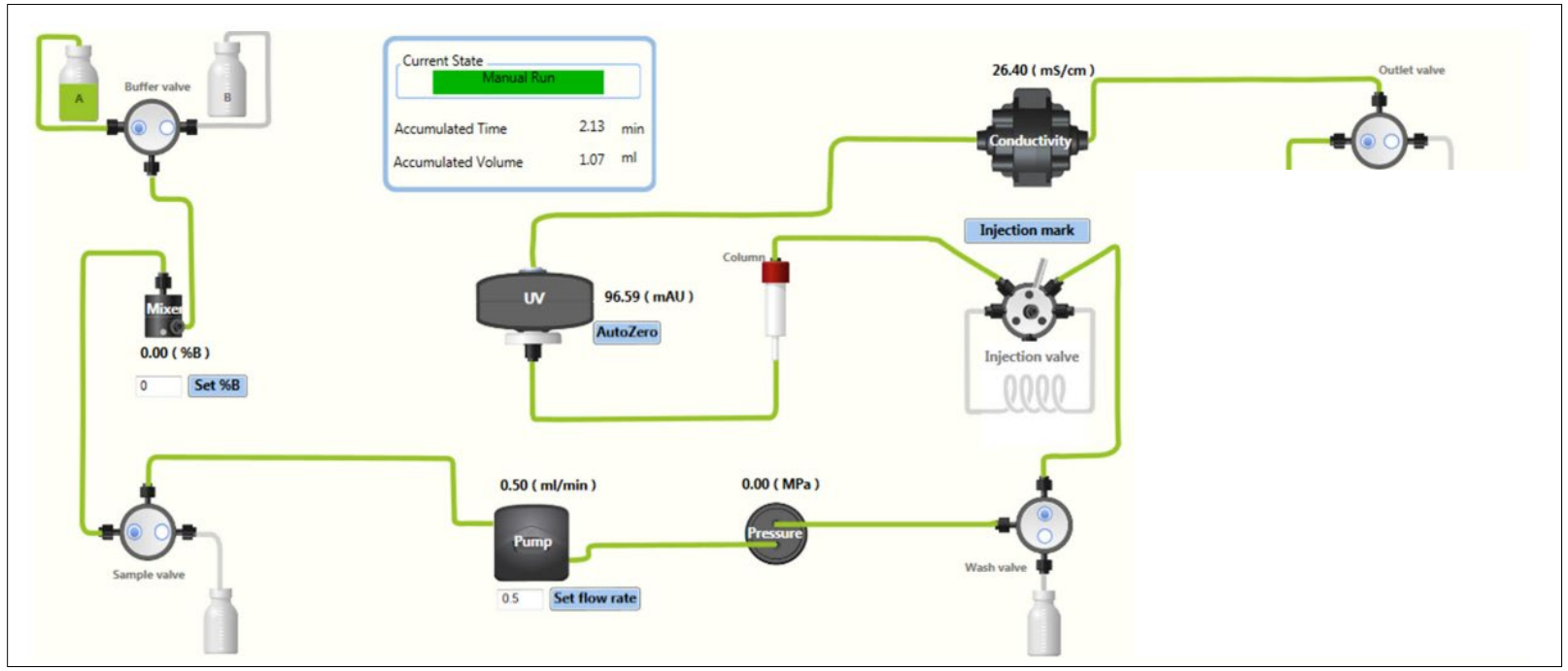




Automated systems – AKTA Start

- Components



Automated systems – AKTA Start

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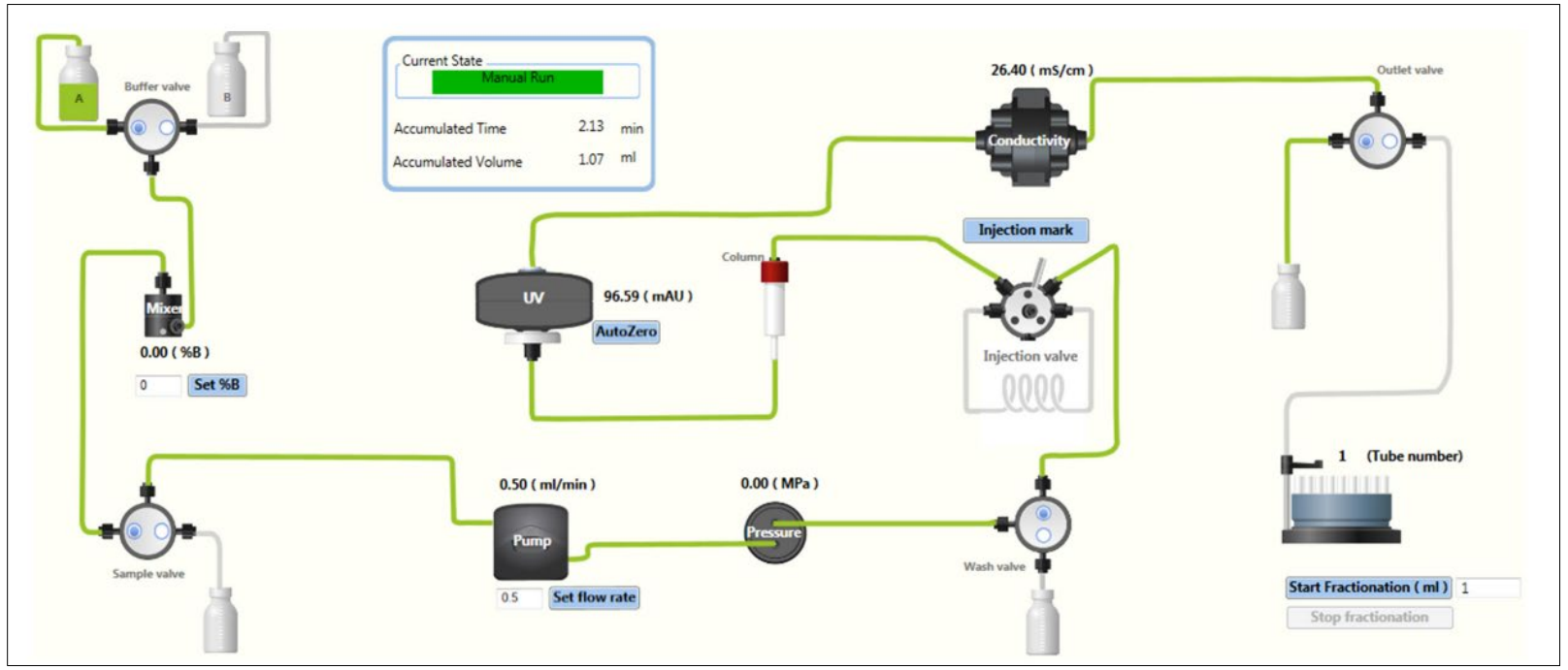
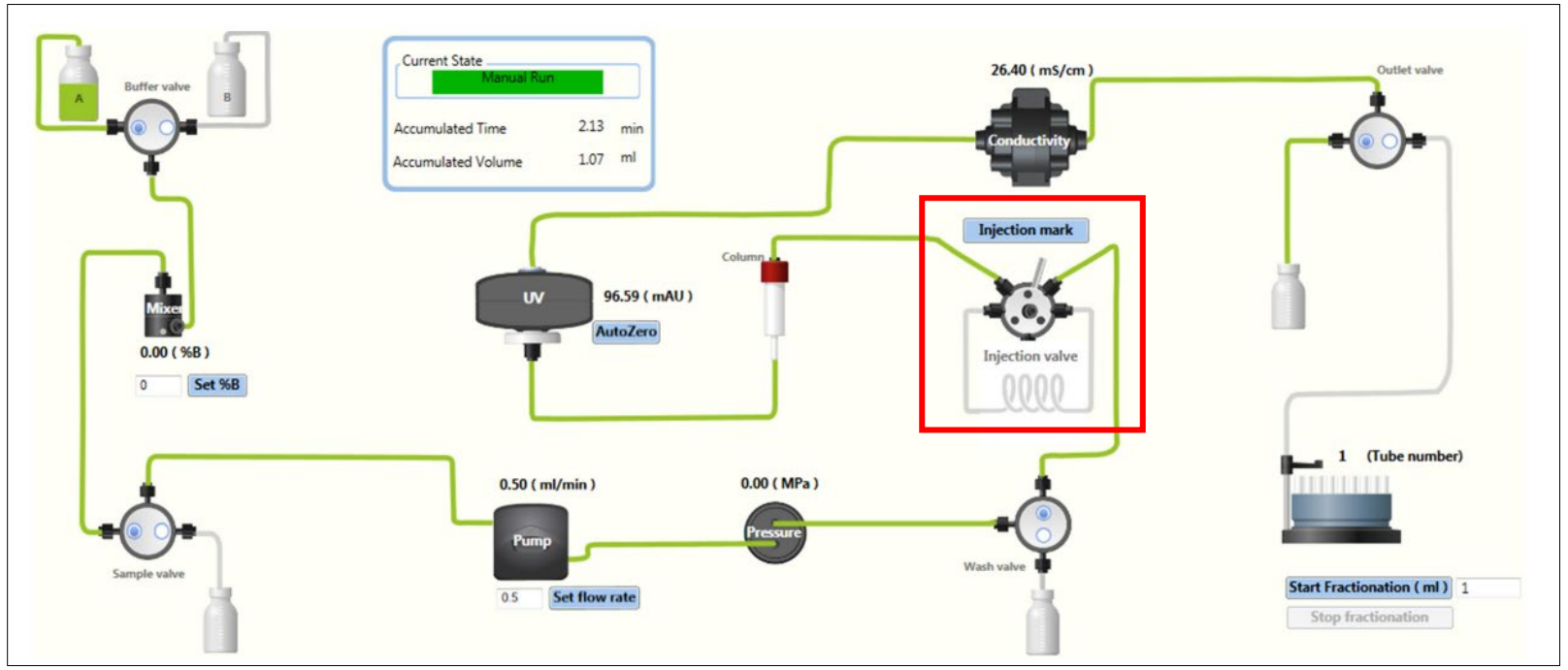
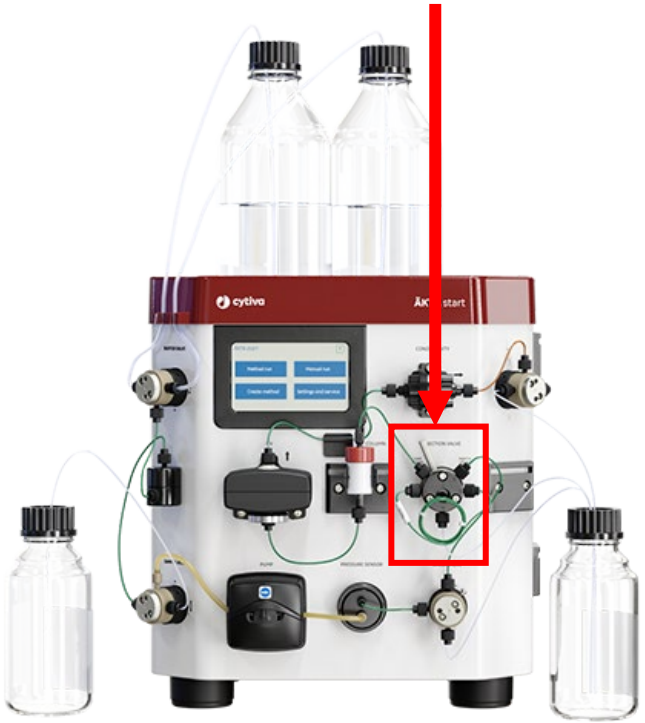


Automated systems – AKTA Start

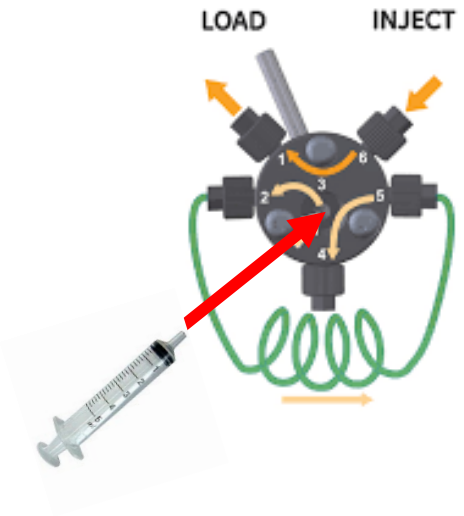
- Sample Injection





Automated systems – AKTA Start

- Sample Injection
  - Your protein sample will be in a syringe in position 3
  - When the valve is set to LOAD, your sample can be loaded into the loop



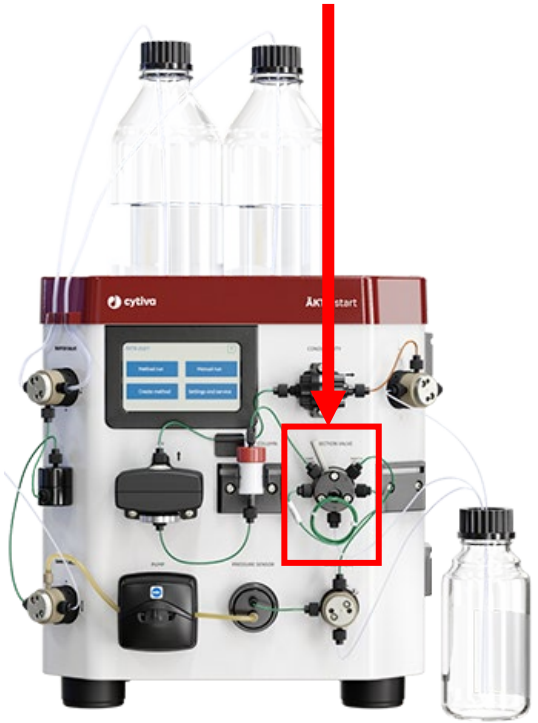
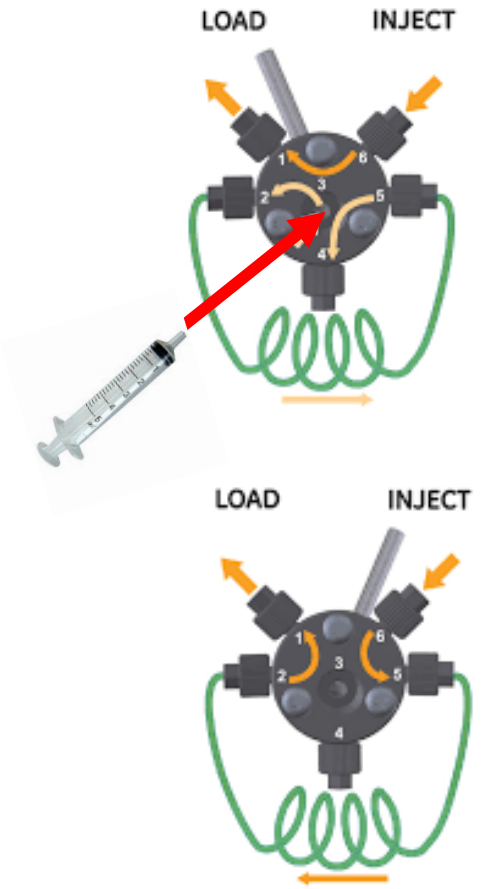


Image:https://bio.pnpi.nrcki.ru/

Automated systems – AKTA Start

- Sample Injection
  - Your protein sample will be in a syringe in position 3
  - When the valve is set to LOAD, your sample can be loaded into the loop
  - When the valve is set to INJECT, your sample (that is now in the loop) will be injected onto the column



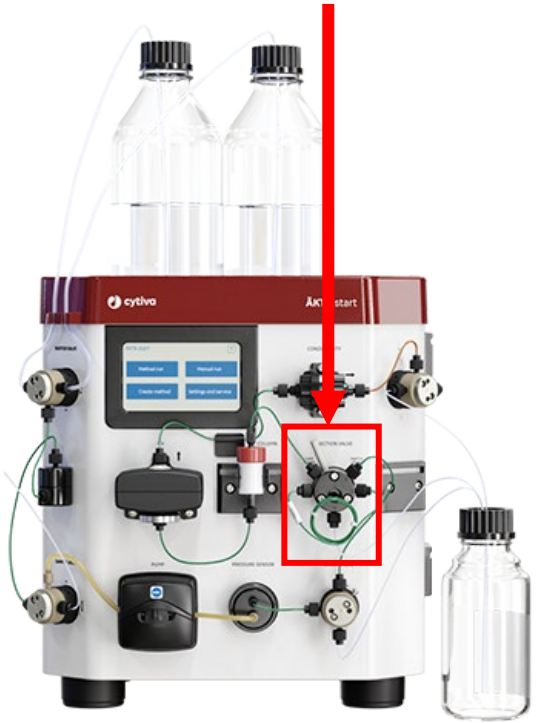
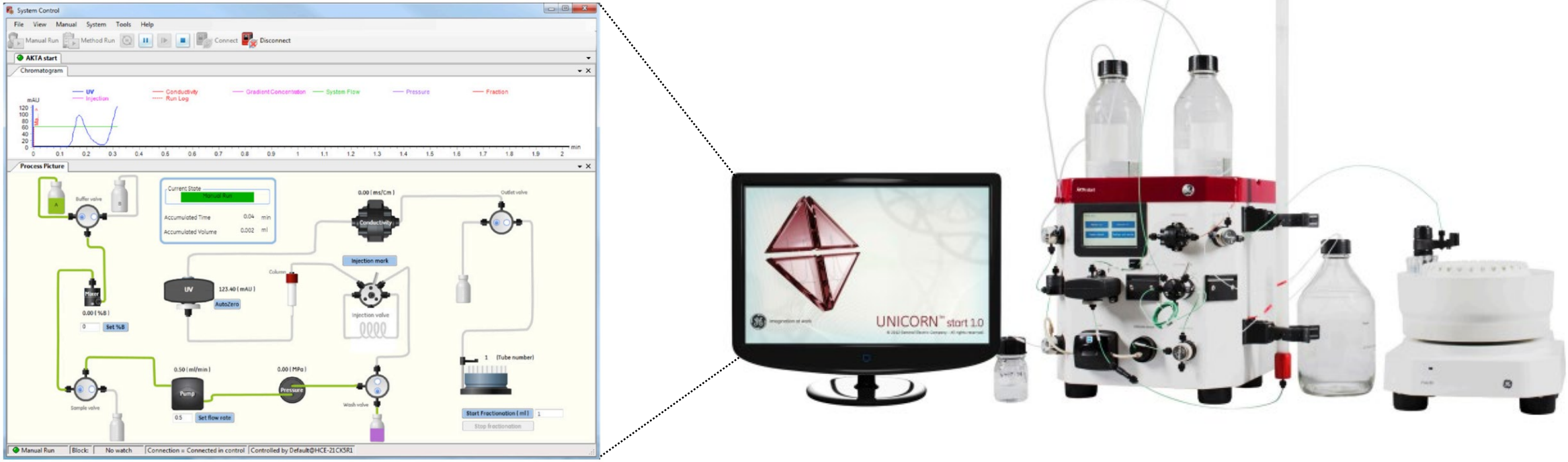


Image:https://bio.pnpi.nrcki.ru/

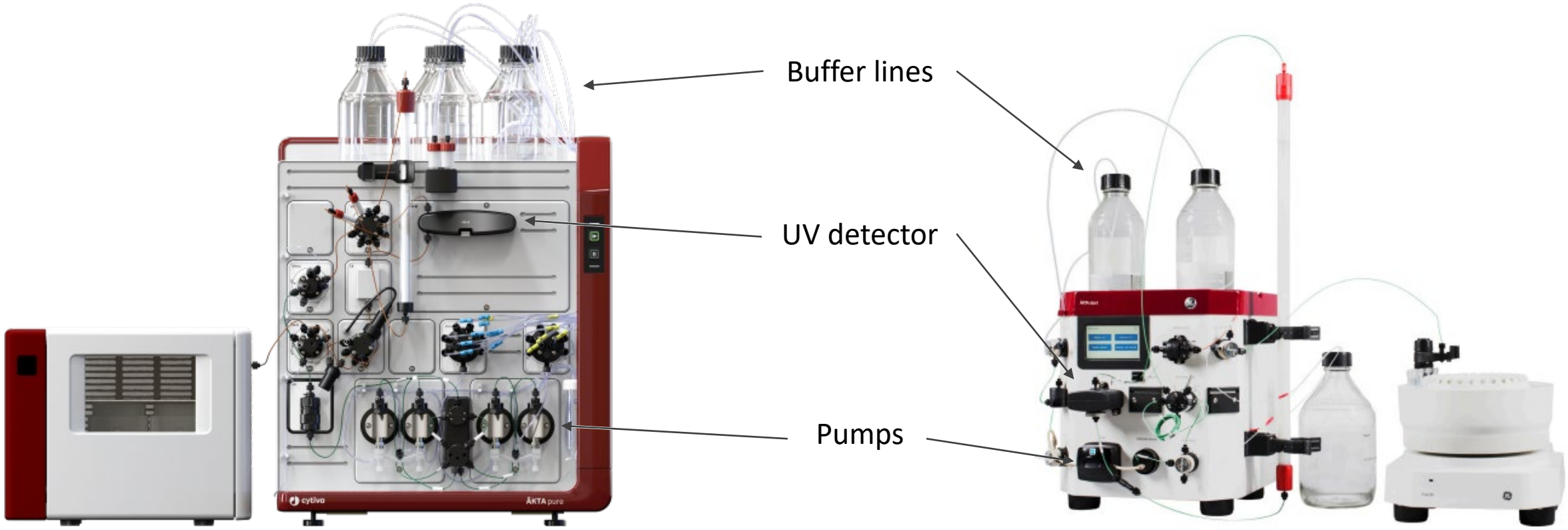
Automated systems – AKTA Start

- Components
  - The system we'll be using has
    - a "carousel" fraction collector
    - computer control

Automated systems – AKTA Start



Automated systems – AKTA Pure



AKTA Chromatography Systems

Start

Go

Pure

Avant

Other Chromatography Systems

Agilent Technologies

Bio-Rad

Shimadzu

Pharmacia
(old AKTA)

Maintaining a chromatography system

- These systems and the associated columns are very sensitive to:
  - Particulates/aggregates

Maintaining a chromatography system

- These systems and the associated columns are very sensitive to:
  - Particulates/aggregates
    - Filter all buffers and samples

Maintaining a chromatography system

- These systems and the associated columns are very sensitive to:
  - Particulates/aggregates
    - Filter all buffers and samples
  - Air bubbles

Maintaining a chromatography system

- These systems and the associated columns are very sensitive to:
  - Particulates/aggregates
    - Filter all buffers and samples
  - Air bubbles
    - Degas buffers, keep bottles topped up
    - Careful protocols when injecting samples

Maintaining a chromatography system

- These systems and the associated columns are very sensitive to:
  - Particulates/aggregates
    - Filter all buffers and samples
  - Air bubbles
    - Degas buffers, keep bottles topped up
    - Careful protocols when injecting samples
  - High pressure

Maintaining a chromatography system

- These systems and the associated columns are very sensitive to:
  - Particulates/aggregates
    - Filter all buffers and samples
  - Air bubbles
    - Degas buffers, keep bottles topped up
    - Careful protocols when injecting samples
  - High pressure
    - Compression of the column resin is bad
    - Include pressure limit settings in protocols

Cleaning columns and safe storage

- After a chromatography run equilibrate your column back into:
  - Sterile-filtered (milli-Q) water, then
  - Ethanol, store in 20% ethanol

- Regularly clean the column with 0.2M NaOH
  - 0.2M NaOH will clean the column but is very corrosive so don’t leave the column in this for any length of time
  - Either inject small volumes or use the Clean-In-Place (CIP) protocols
  - For this see your handbook or the relevant column datasheet

Protein Purification – Part 2

- Today we will learnt about:
  - Other chromatography techniques
    - Size-exclusion
    - Ion exchange
    - HIC and others
  - Chromatography equipment
    - The components and how to inject sample
    - The dos and don’ts of good chromatography

Questions?

Then let’s do some SEC