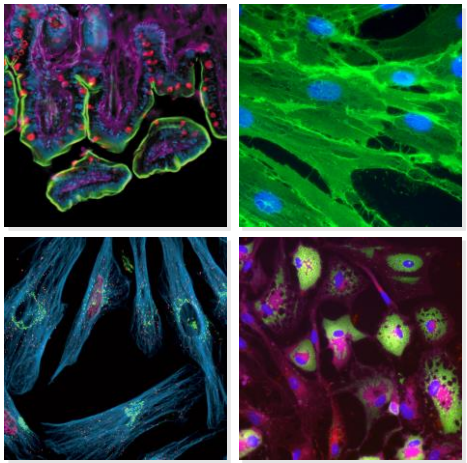

Case Studies of POROS Chromatography for Use in Vaccine and Large Biomolecule Purification

Rev 1 (Life Tech template)

Shelly Cote Parra

April 2011

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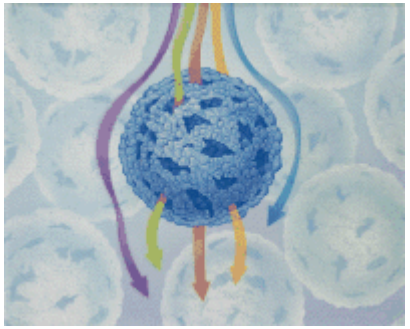
Topics

- Introduction to POROS® Chromatography Products
  - Principles, Product Attributes & Advantages
- Applications
  - POROS® XS: New Cation Exchanger
  - POROS® Anion Exchangers
  - Go-Pure™ Pre-packed Chromatography Columns
  - Purification of Large Biomolecules
- Future Products & Product Development
- Closing Remarks



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Purification of Large Biomolecules: Viruses, VLP, Vectors, Plasmid DNA, IgM, Fusion Proteins

- Why is POROS suited for these applications?
  - Unique pore morphology which increases surface area rendering large biomolecules accessible to charged functional groups
  - Ultimately increasing capacity for large biomolecules
  - Ability to flow at high flow rates makes the process more flexible, scalable and productive while maintaining resolution
  - Easy to use and pack
  - Reproducible

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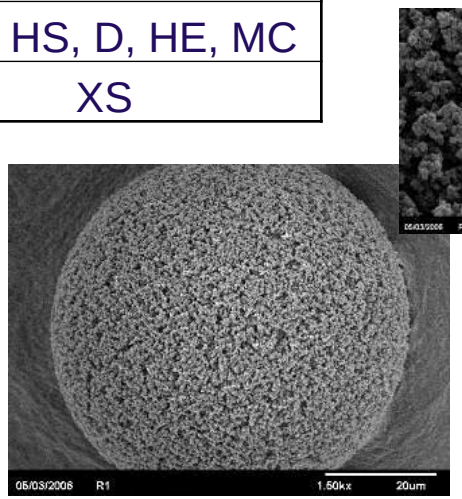
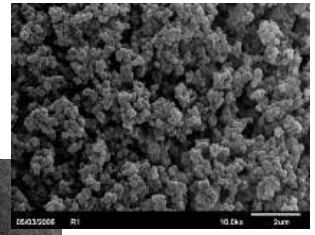
Purification of Large Biomolecules

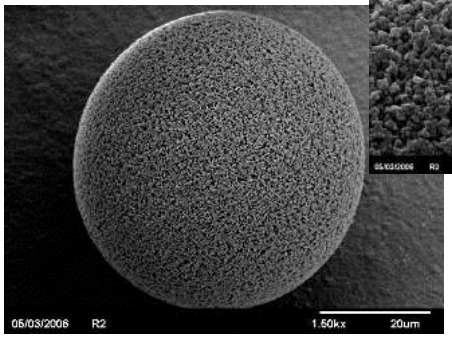
Base Bead	Average Pore Size (Angstroms)	Average Pore Size (nm)	Functional Chemistries
R150	3600	360	HQ, PI
R250	1600	160	A50, HS, D, HE, MC
R550	1100	110	XS

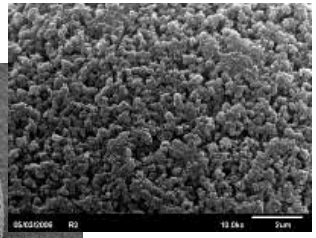




R150

- Smallpox 200-400nm in diameter
- Adenovirus 70-90nm in diameter
- Adeno-associated virus 20nm in diameter
- Papilloma virus 40-55nm in diameter
- IgG, 150kDa, Molecular radius = 55 Angstroms
- IgM, 900kDa, Molecular radius = 120 Angstroms
- Bacterial Polysaccharides = 500-1000kDa
- Plasma DNA ~10^6 Da, radius differs based on configuration





R250

POROS® Applications: Purification of Viruses, Virus-Like Particles (VLPs), Vectors, and Plasmid DNA

- Known applications for large biomolecule purifications center around 5 POROS Chemistries: HS50, HQ50, D50, PI50, R150
- Particles bind tightly as elution is typically at 0.4->1.0M NaCl
- Can be employed as capture or polish chromatography step
- High purity achieved in one chromatographic step

Case Study 1

- Cook, James C. et al, “Purification of Virus-Like Particles of Recombinant Human Papillomavirus Type 11 Major Capsid Protein L1 from *Saccharomyces cerevisiae”, Protein Expression and Purification,* 1999, V 17, Pages 477-484

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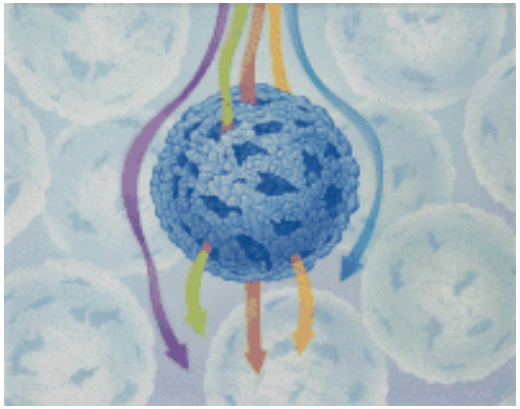

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Case Study 1: Purification of HPV VLPs

- Single Step Chromatographic Purification

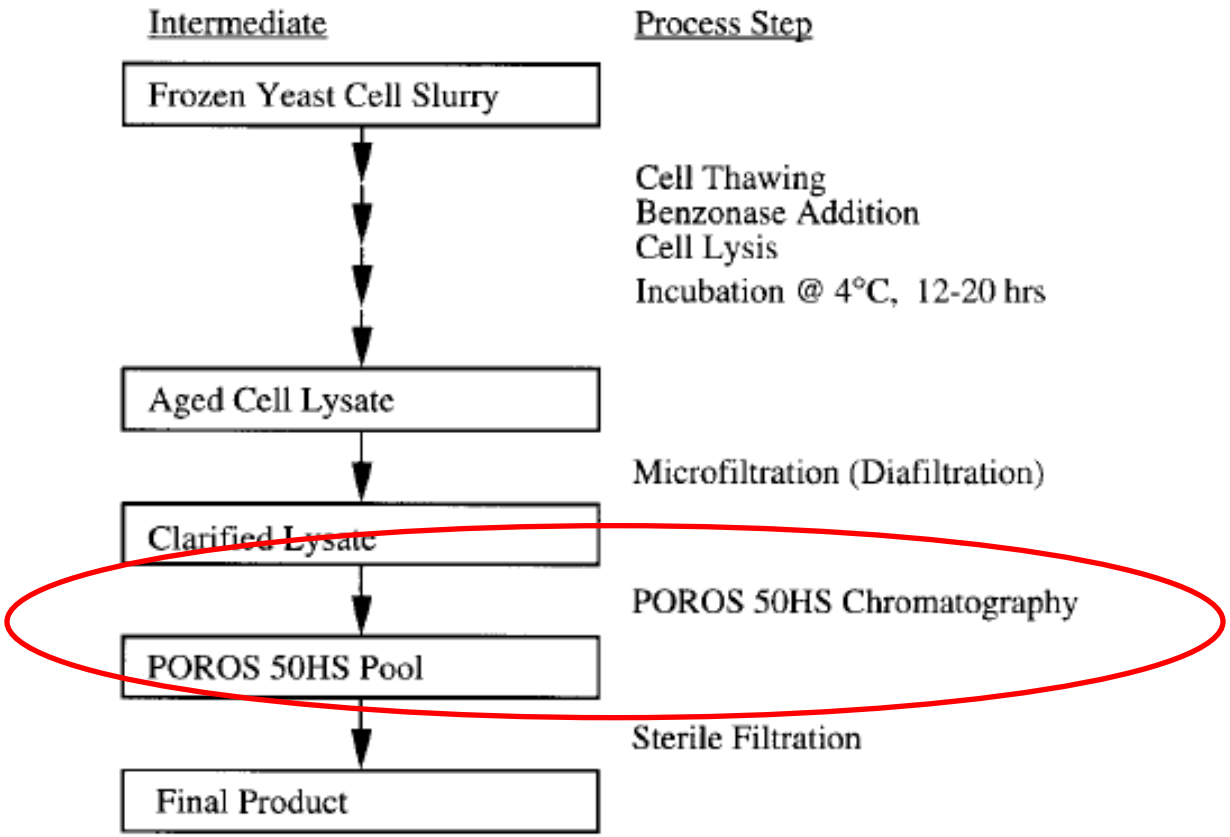

FIG. 2. Overview of purification process.

Reprinted from Cook, J., *Protein Expression and Purification*, 1999, V17, 477-484

Case Study 1: Purification of HPV VLPs

- Single Step Chromatographic Purification
  - Column: 14cmDx10cmL (1.5L)
  - Flow rate: 50cm/hr
  - Bind in 0.2M MOPS/ 0.4M NaCl, pH 7.0
  - Washed with 50mM MOPS/ 0.5M NaCl, pH 7.0
  - Elute in 10 CV gradient, 0.5M NaCl to 1.5M NaCl
  - Elution at ~1.1M NaCl
  - 98% Pure by SDS-PAGE
  - Monomeric form maintained

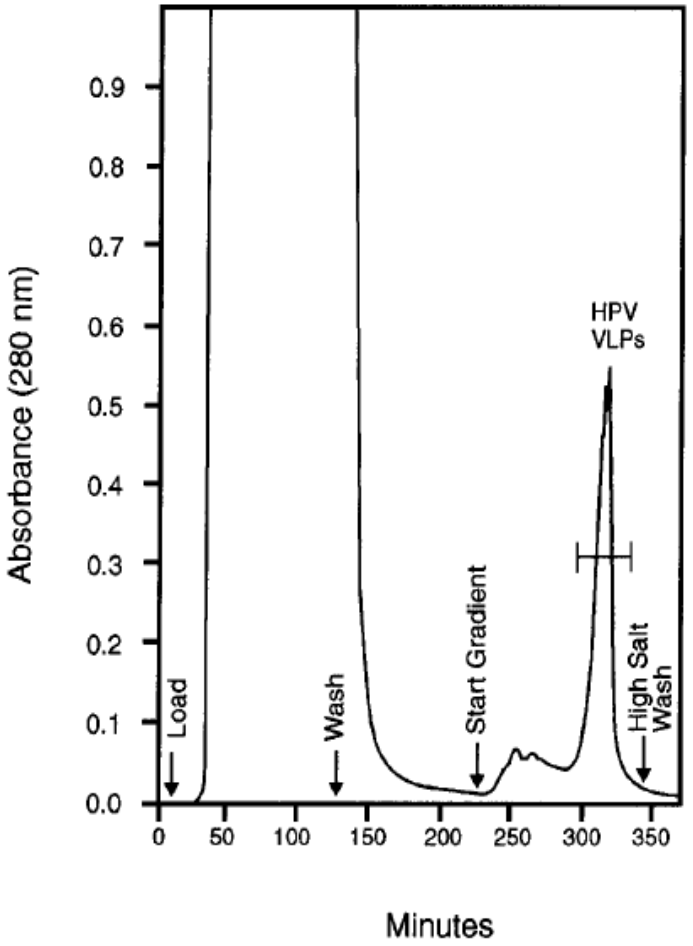

FIG. 1. Typical POROS 50HS chromatogram of the clarified lysate. Elution with a linear NaCl gradient caused the antigen to elute between 0.9 and 1.35 M NaCl with the peak eluting at 1.06 M NaCl.

Reprinted from Cook, J., *Protein Expression and Purification*, 1997, V17, 477-484

Case Study 1: Purification of HPV VLPs

- Advantages of POROS HS50
  - Pore Structure
    - > “The large throughpores of POROS 50HS resin were probably accessible to the VLP,…”
    - > “Small pores would physically exclude the VLP and restrict binding to the outer surface of the beads, thus leading to low binding capacity.”
  - Selectivity and Recovery
    - > “Excellent selectivity of the chromatography made it possible to achieve 98% purity” with a 2 step purification process: microfiltration and CEX Chromatography
    - > POROS HS50 was “the best for selectivity and recovery for this particular application”

Case Study 1: Purification of HPV VLPs

- Thoughts for Improving Recovery
  - Elute in reverse-flow mode
  - Utilize step elution versus gradient elution
  - Increase salt concentration, > 1.06M, as 0.5M NaOH is required to remove contaminants for complete regeneration

Case Study 2

- Qu, Guang, et al, “Separation of Adeno-Associated Virus Type 2 Empty Particles from Genome Containing Vectors by Anion-Exchange Column Chromatography”, *Journal of Virological Methods*, 2007, V 140, Pages 183-192
- Work completed by Avigen and Children’s Hospital of Philadelphia

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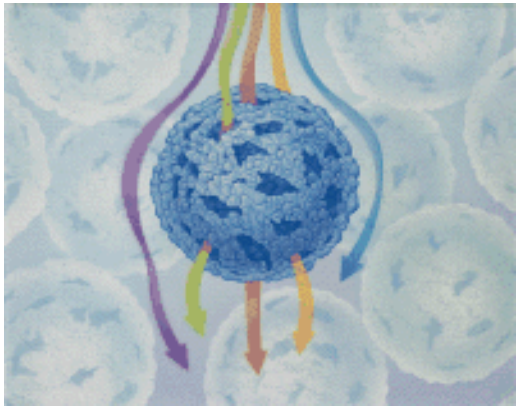

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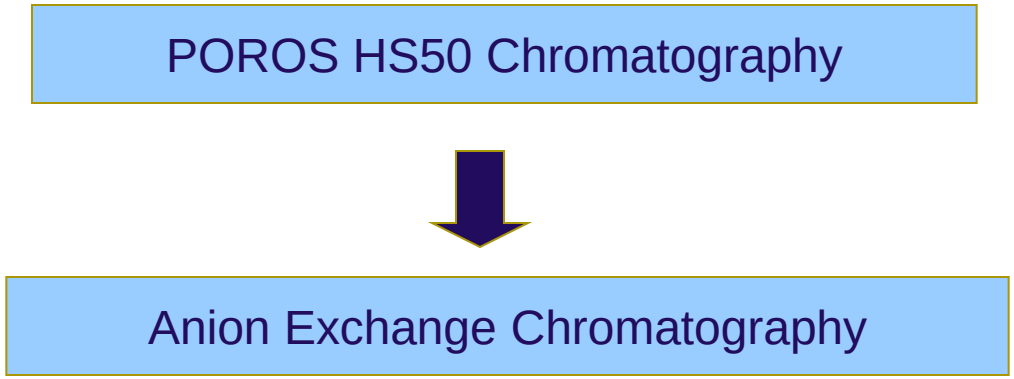
Case Study 2: Separation of AAV Empty Capsids from Genome Containing Vectors

- Adeno-Associated Virus (AAV) vectors are small, non-enveloped single-stranded DNA viruses used in gene therapy and vaccination
- Benefits of AAV vectors
  - Stable, long-term transgene expression
  - Cause mild immune response
  - Ability to infect both dividing and non-dividing cells and tissues
  - Site specific integration
- Disadvantages
  - Limited genetic capacity
  - Production of these vectors has been known to be labor-intensive, difficult to scale-up and expensive
  - Empty capsids are a byproduct of vector biosynthesis

Case Study 2: Separation of AAV Empty Capsids from Genome Containing Vectors

- 2 Step Chromatographic Purification Process



Case Study 2: Separation of AAV Empty Capsids from Genome Containing Vectors

- 2 Step Chromatographic Purification Process

Step 1 | POROS HS50 Chromatography

- Equilibrated with 20mM $NaP0_4$/100mM NaCl, pH 7.4 at 150 cm/hr
- Washed with 3 CV equil buffer
- Washed with 5 CV 20mM $NaP0_4$/100mM NaCl/5mM Sarkosyl, pH 7.4
- Washed again with 3 CV equil buffer
- Eluted with 20mM $NaP0_4$/370mM NaCl, pH 7.4
- Eluted both vector particles and empty capsids, but “substantially free of other impurities”

Case Study 2: Separation of AAV Empty Capsids from Genome Containing Vectors

- POROS HS column was developed to remove nucleic acids that can cause particle aggregation

- Redesigned HS step:
  - Previously purified HS pool was diluted 2 fold with 20mM $NaP0_4$, pH 7.4 and loaded onto HS column
  - Nuclease digestion:
    - > Washed with 5 CV 20mM $NaP0_4$/100mM NaCl/2mM $MgCl_2$, pH 7.4 containing 200 units/ml Benzonase and 10 units/ml DNase 1
    - > Enzymes added at 150 cm/hr for 1 CV and 30 cm/hr for 2 CV
  - Washed with 4CV 20mM Tris-Cl/480mM Ammonium Acetate, pH 8.5
  - Eluted with 3CV 20mM $NaP0_4$/600mM Ammonium Acetate, pH 8.5

Case Study 2: Separation of AAV Empty Capsids from Genome Containing Vectors

- 2 Step Chromatographic Purification Process Development

Step 2 | Anion Exchange Chromatography

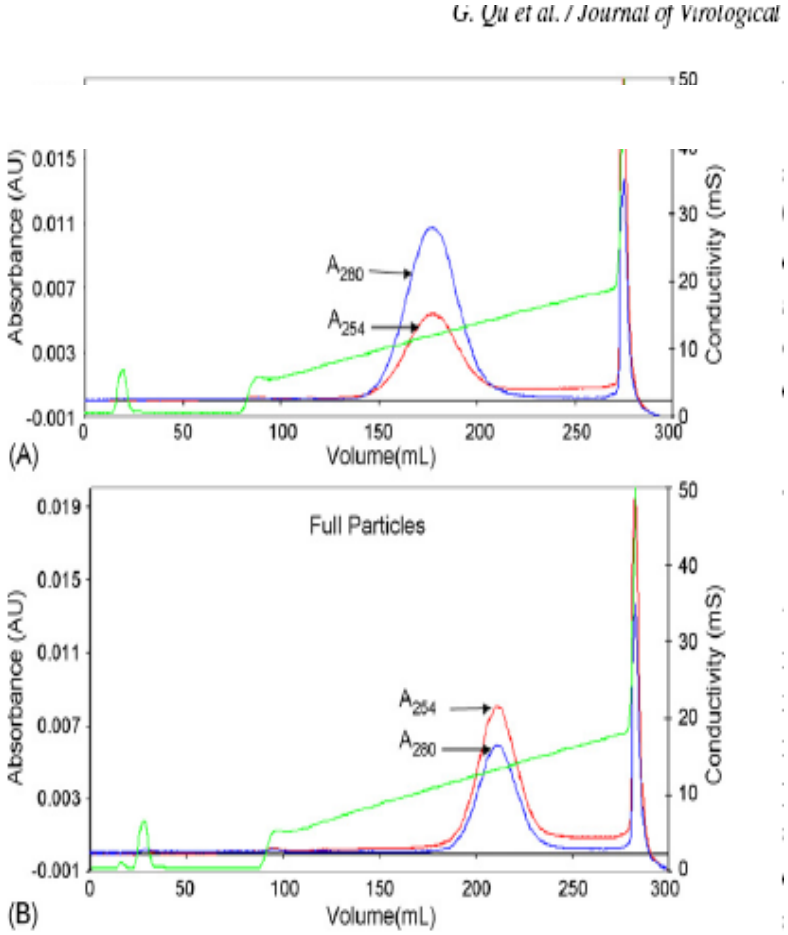
- Tested 8 different AEX medias including POROS HQ, D, and PI for ability to bind and elute
- Initial column conditions
  - Equilibrated 3 ml column with 10mM $NaP0_4$/50mM NaCl, pH 7.4
  - 30 ml clarified lysate was diluted 4 fold with 20mM $NaP0_4$, pH 7.4 and loaded on each column
  - Wash with 10 ml equil buffer
  - Eluted with 10 ml 20mM $NaP0_4$/1M NaCl, pH 7.4

Case Study 2: Separation of AAV Empty Capsids from Genome Containing Vectors

- All medias were able to bind and elute the desired AAV capsid
- Further development was performed using 2 selected resins: POROS HQ50 and 1 other Q resin
  - These were determined to be “most readily adaptable to large scale chromatography in a GMP environment”
  - POROS HQ50 was used for all the initial AEX development work

Case Study 2: Separation of AAV Empty Capsids from Genome Containing Vectors

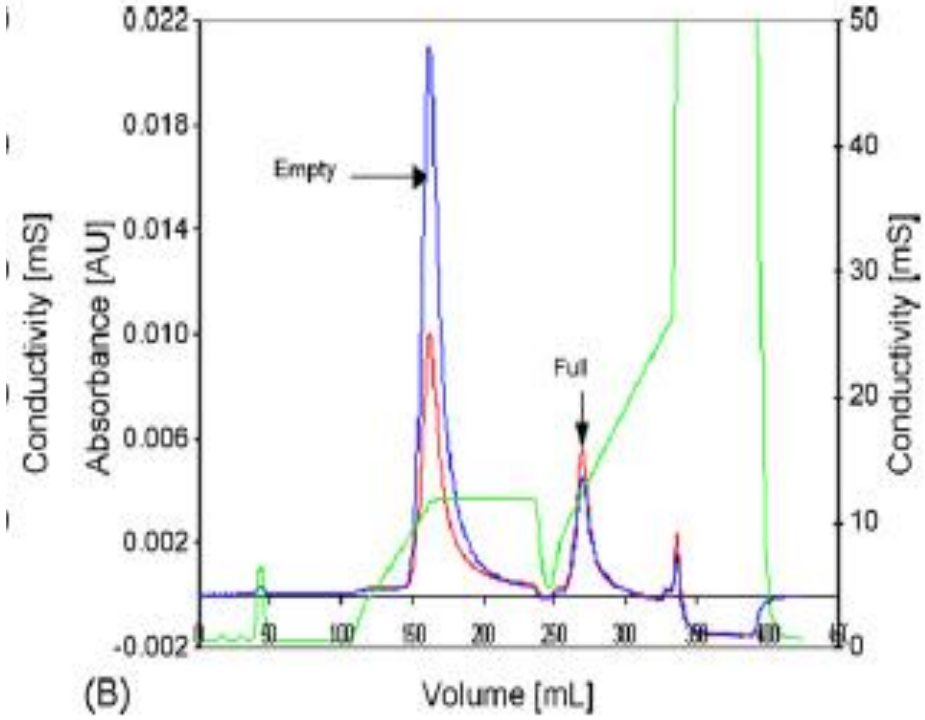
- Slightly different anionic behavior was shown with pre-purified empty capsids and full vectors using POROS HQ
  - Empty peak eluted at ~175 mM NaAc
  - Vector peak eluted at ~215 mM NaAc
- Full vectors bound more tightly to the AEX media compared to the empty capsids suggesting they are more negatively charged
- Chromatography conditions:
  - Equilibrated/loaded in 20mM Tris-Cl/50mM NaAc, pH 7.4
  - Washed with 3 CV equil buffer
  - Elution: 10 CV linear gradient from 50-300 mM NaAc



Reprinted from Qu, Guang, *Journal of Virological Methods*, 2007, V140, 183-192

Case Study 2: Separation of AAV Empty Capsids from Genome Containing Vectors

- POROS HQ was able to efficiently separate a spiked mixture of empty capsids and full vectors (16:1 ratio)
- Chromatography conditions:
  - Equilibrated in 20mM Tris-Cl/50mM NaAc, pH 8.0
  - 3 CV linear gradient from 0-165 mM NaAc
  - 2 CV 165 mM NaAc Wash
  - Elution: 5 CV linear gradient from 50-300 mM NaAc
  - Strip with 1M NaCl



Reprinted from Qu, Guang, *Journal of Virological Methods*, 2007, V140, 183-192

Case Study 3

- Development of Methods for Large-Scale Plasmid DNA Purification
- Performed by Paul Lynch at Applied Biosystems

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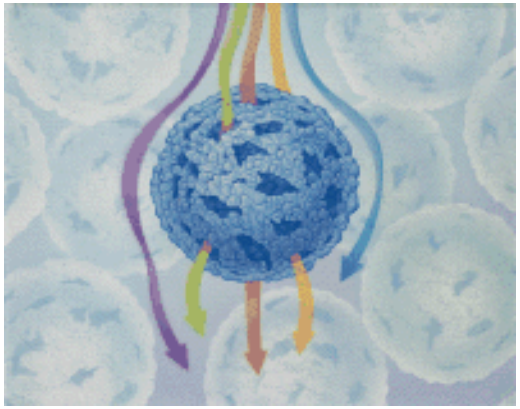

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Case Study 3: DNA Plasmid Purification

- Plasmid DNA has been used in both vaccination and gene therapy applications since 1992
- Plasmids are usually circular, double stranded, DNA molecules
- Advantages
  - Safer, non-pathogenic with few side affects
  - Specific immune response
  - Can express multiple antigens in a single plasmid
  - Ease to design, develop and manufacture
  - Cost effective
  - Stable for storage and shipment
- Disadvantages
  - Can have a slow immune response
  - Limited effectiveness shown in human clinical trials
  - Possible integration into human cellular DNA
  - Public acceptance

Case Study 3: DNA Plasmid Purification

- Plasmid Purification Challenges
  - Large molecules → low binding capacity
    - > Binding normally occurs only on the outside of the bead
  - Contaminants with similar properties
    - > *e.g.* Genomic DNA/RNA
  - Viscosity at high concentrations
  - Supercoiled *vs* linear

Case Study 3: DNA Plasmid Purification

- Benefits of POROS for Plasmid Purification and PD Suggestions
  - ↑ Capacity by using smaller beads to increase surface area in a column (POROS ~50 µm)
  - Larger throughpores on POROS HQ and PI can potentially increase capacity and improve mass transfer
  - Due to the high viscosity of product loads, POROS allows for productivity to remain high since flow rates do not need to be decreased
  - Cationic compaction agents such as polyamines (i.e. spermidine and spermine), PEG, alcohol and ammonium sulfate can change the confirmation of the DNA molecule to increase capacity and allow easier access to the pores
    - > This conformational change has also been found to increase resolution, yield and selectivity

Case Study 3: DNA Plasmid Purification

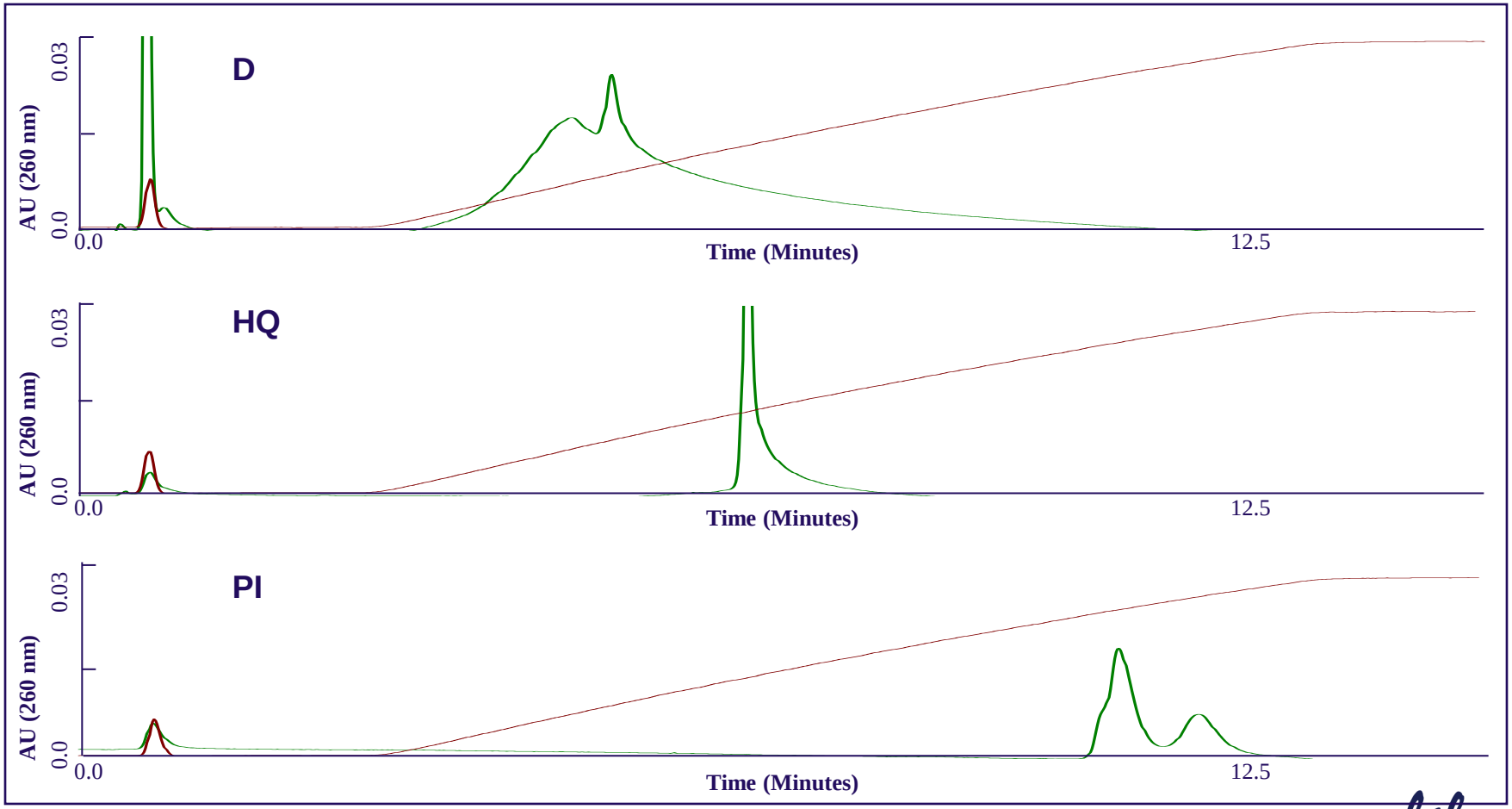
- AEX most used technique for purifying plasmid DNA
- DNA binds to an AEX column usually >pH 4.0 since the molecule becomes negatively charged
  - Running at pH 7.0-8.0 is common on AEX columns
- Load should be >0.5M NaCl to cause low charge density impurities to flow through
- DNA is eluted with a salt gradient
- Goals of DNA plasmid purification study
  - Determine binding characteristics
    - > Screen anion exchange chemistries
    - > Uses purified plasmid
  - Develop a scalable purification method

Case Study 3: DNA Plasmid Purification

- Screen chemistries with purified plasmid
  - POROS D - new weak anion exchange
  - POROS HQ - strong anion exchanger
  - POROS PI - weak anion exchanger
- Screening Operating conditions:
  - Columns: 4.6 mmD x 100 mmL (1.66 ml)
  - Flow rate: 1000 cm/hr (3ml/min)
  - Equilibration: 50mM Tris, 0.2M NaCl, pH 7.0
  - Gradient Elution: 0.2 - 2M NaCl over 15 CV, pH 7.0

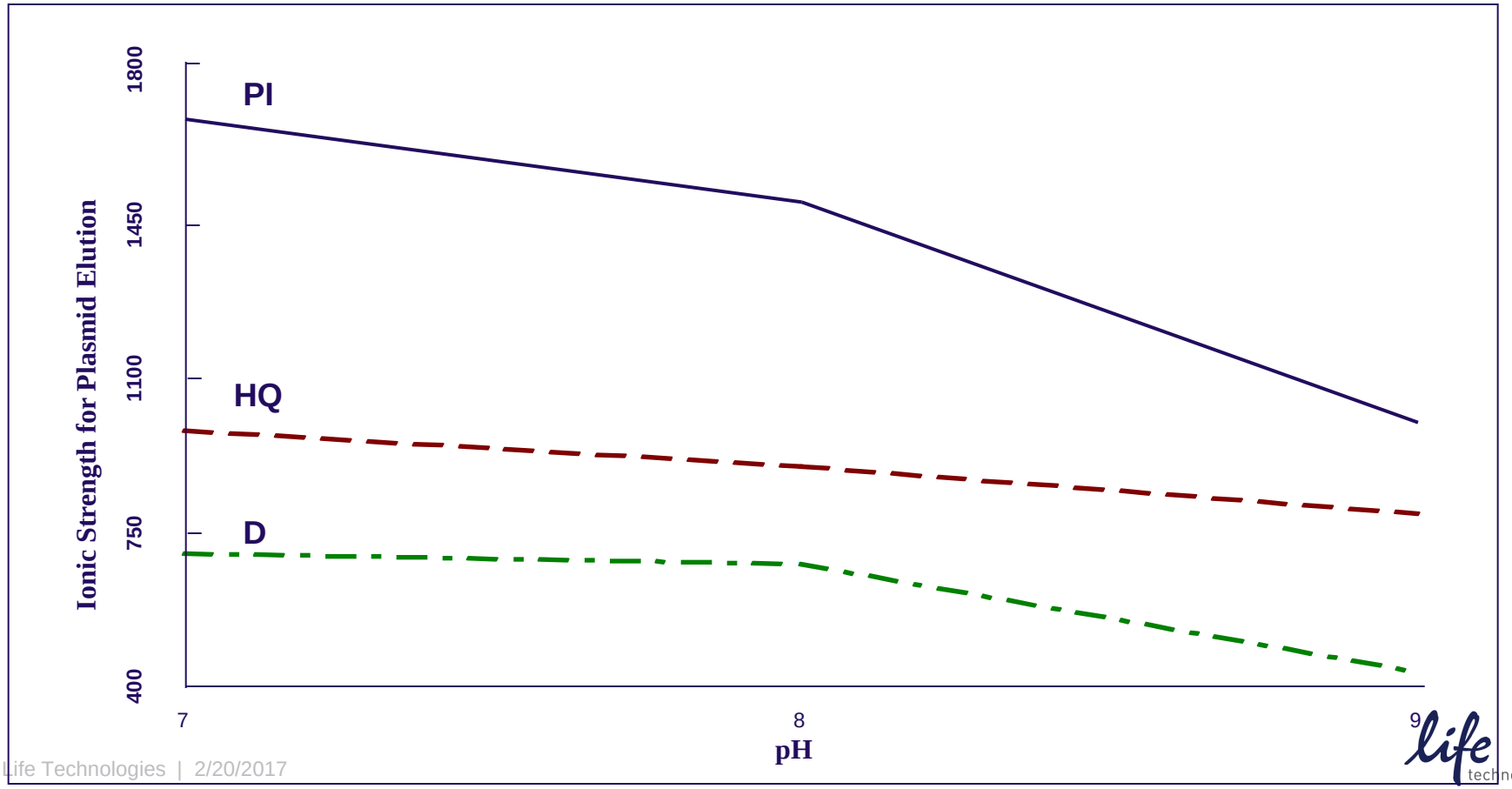
Case Study 3: Chemistry Screening at pH 7.0

The plasmid bound tightest on POROS PI and eluted at ~ 1.6M NaCl

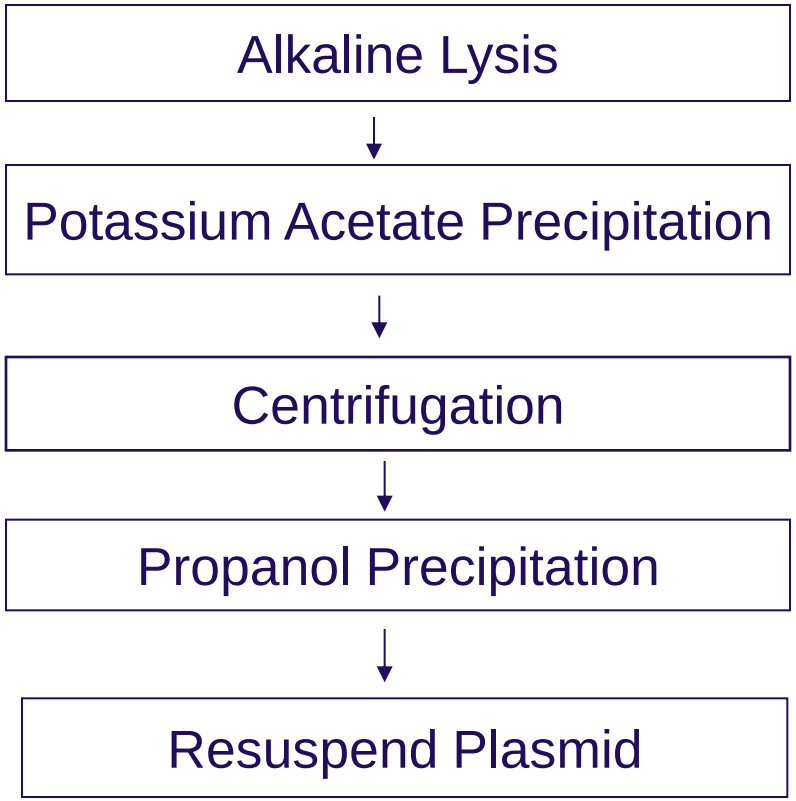


Case Study 3: Effect of pH on Chemistry Screening

PI50 media had the strongest retention of plasmid allowing for a high salt process in which many contaminants will flow through. Further development performed on POROS PI50.

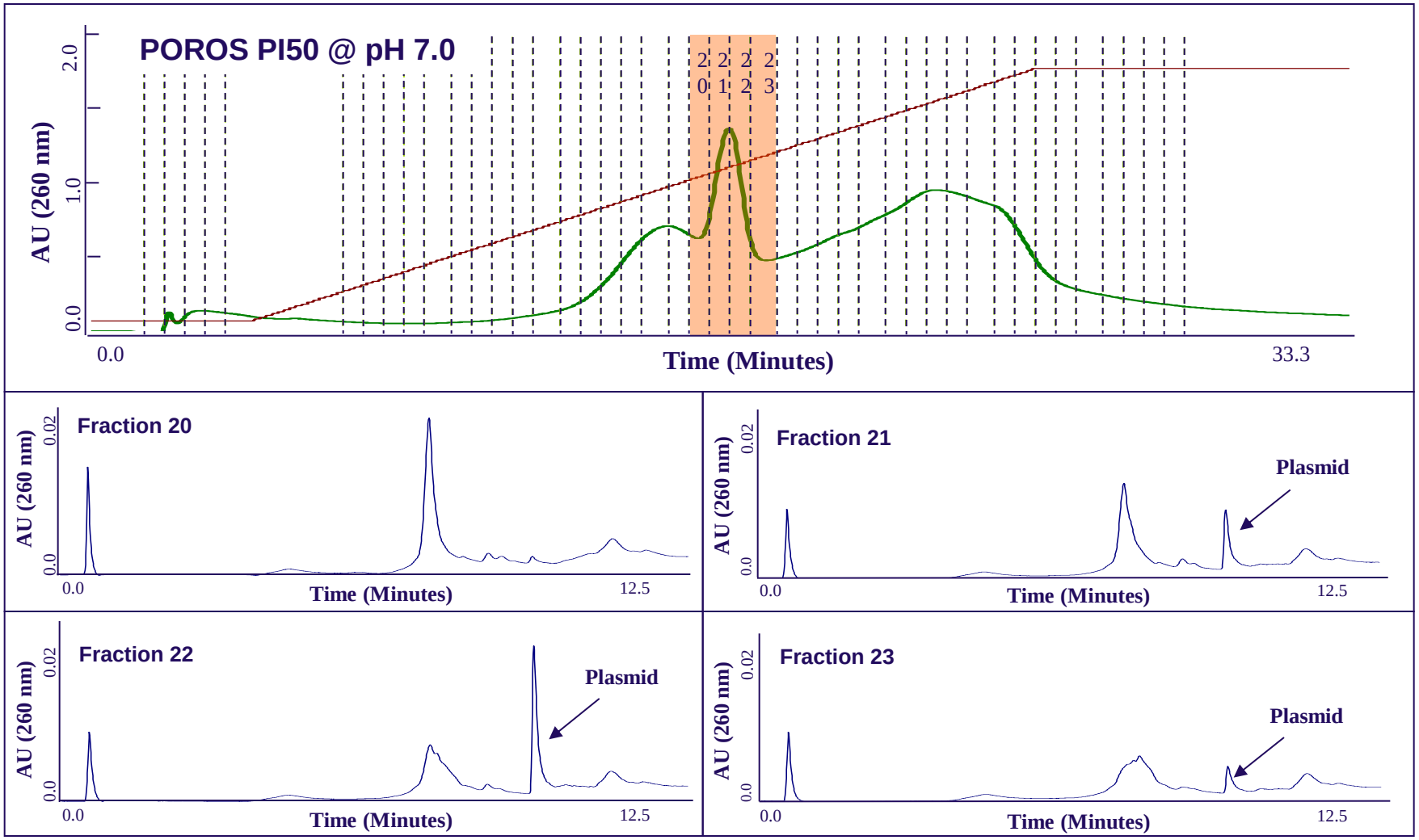


Case Study 3: Sample Preparation Flow Diagram



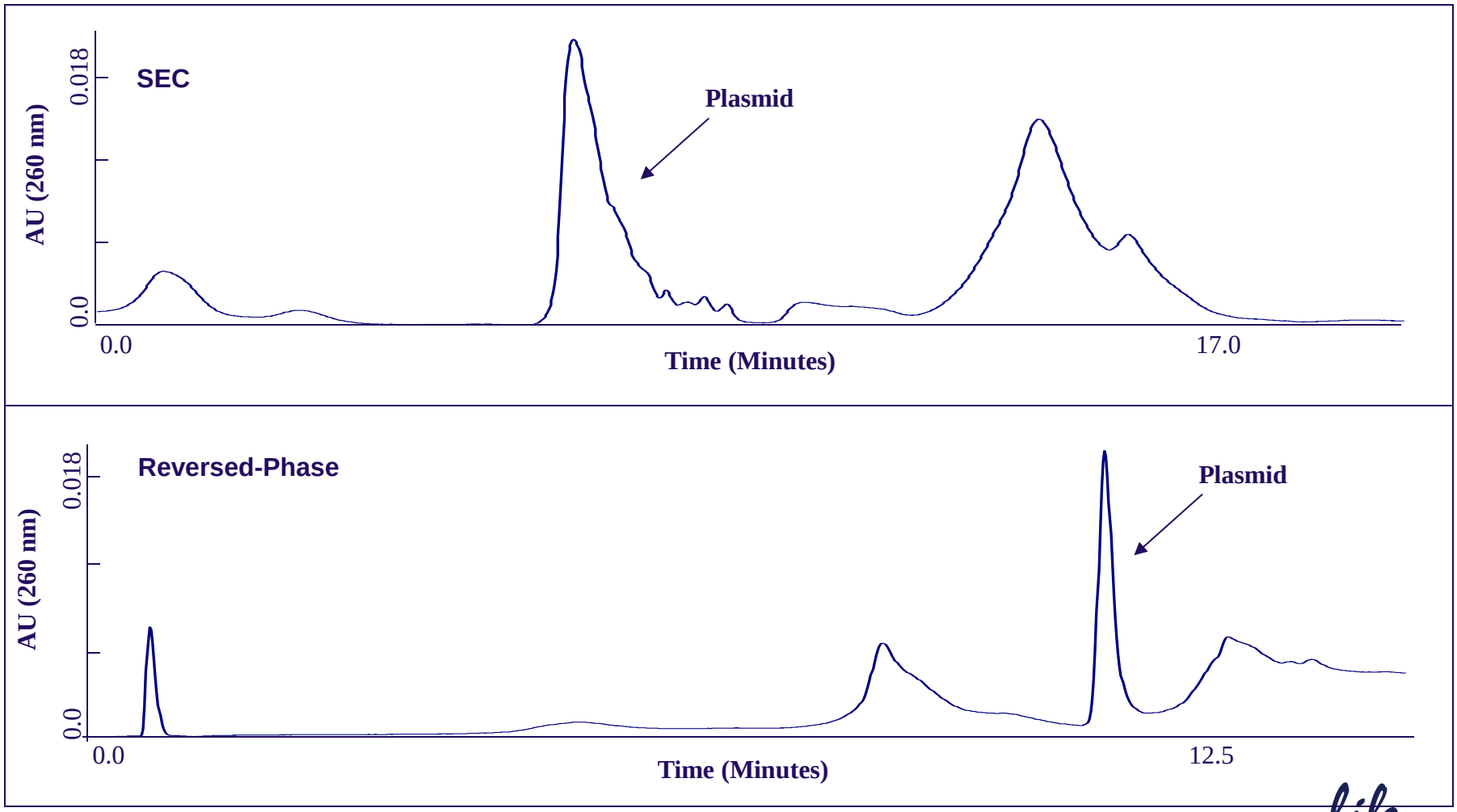
Case Study 3: RP Analysis of POROS PI50 at pH 7.0

The DNA plasmid elutes from the reverse phase column in fractions #21-23



Case Study 3: Assays of PI Fractions 21 to 23 Pool

Integration of analytical methods shows that the plasmid peak is 40% pure

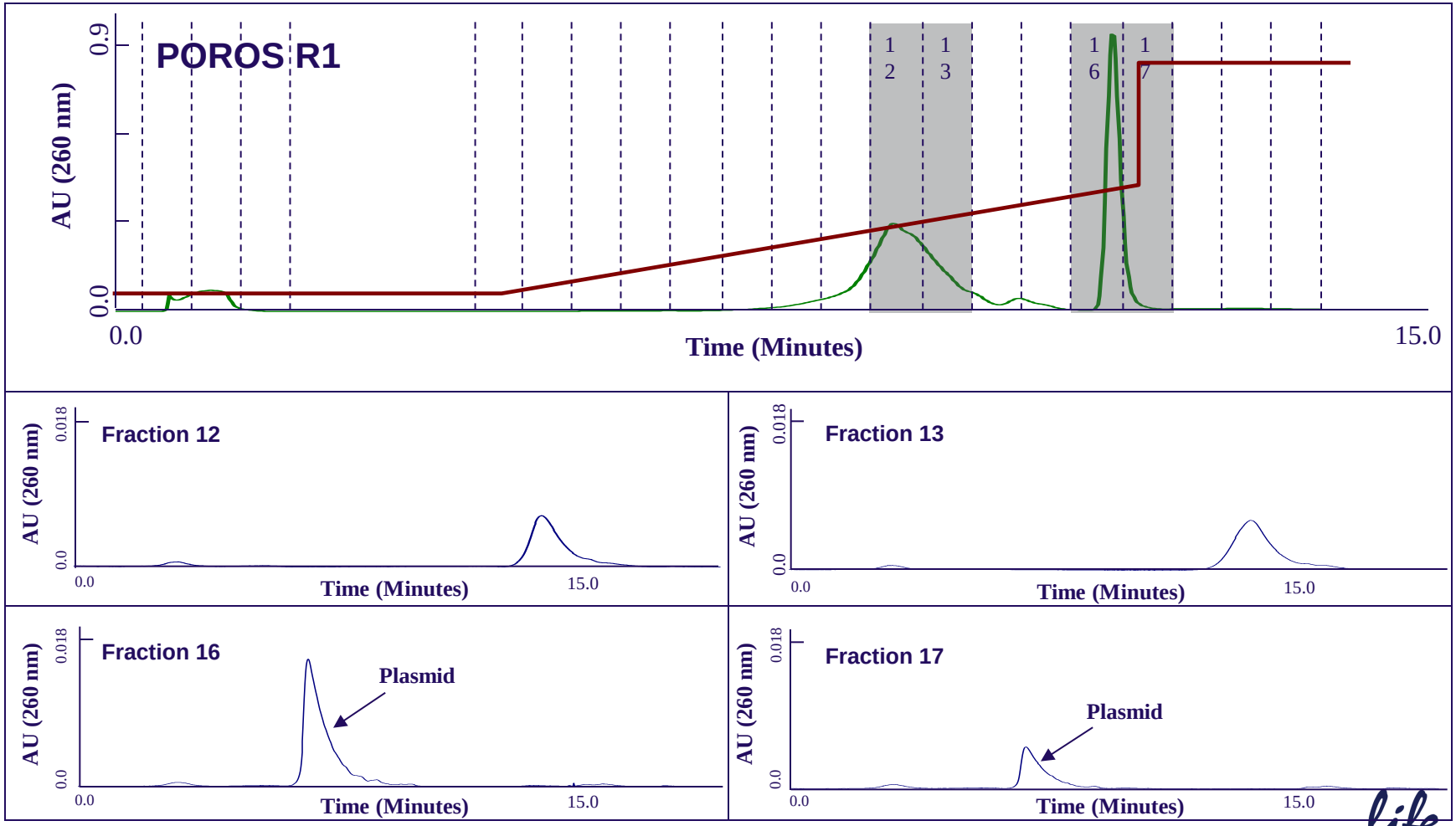


Case Study 3: Anion Exchange Summary

- Separation optimized and scaled-up on POROS PI50, pH7.0
  - Flow rate: 1000 cm/hr (3mls/min)
  - Equilibration: 50mM Tris, 1.2M NaCl, pH 7.0
  - Gradient Elution: 1.2 - 2M NaCl over 15 CV, pH 7.0
- Pooled fractions 21-23
- Size exclusion and reversed-phase fraction analysis
  - Plasmid was 40% pure using both techniques.
  - Analytical results agreed with gel analysis (not shown)
- Second step needed to remove remainder of contaminants

Case Study 3: SEC Analysis of POROS R1 Step

The DNA plasmid elutes from the reverse phase column in fractions #16-17 and is >98% pure



Case Study 3: Reversed-Phase Summary

- Second step developed on POROS R150
  - Flow rate: 1000cm/hr (3mls/min)
  - Buffers:
    - A -100mM TEAA (Triethyl ammonium acetate)
    - B - 10%TEAA/Acetonitrile
  - Equilibration: 100% buffer A
  - Gradient Elution: 0 - 20%B over 15 CV
- Can be performed on 10, 20, 50 µm POROS R1
  - Permitted use as a preparative or analytical technique
- POROS polystyrene RP media can be run under basic conditions
  - Not possible with silica based reversed-phase media
- Separated remaining DNA/RNA contaminants
- SEC analysis showed plasmid purity greater than 98%

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