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Key Issues in the Process Development of Purification Procedures for Viral Vector and Plasmid Biopharmaceuticals

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With the advent of the first gene therapy product to market, the industry faces the challenge of mass-producing high-purity viral particles and plasmids. The concept of manufacturing therapeutic genes rather than therapeutic proteins as marketable products is still in its infancy. Although manufacturers of biopharmaceuticals have decades of experience in the purification of proteins, virus and plasmid products pose unique challenges that cannot be addressed without some modifications to traditional, protein-based approaches.

Table 1a. Effects of bead diameter on dynamic binding capacity at 10% breakthrough (Q10) at 150 cm/h and 5 cm bed height for a ligand S-aryl media. A pre-cleared, group separated alkaline lysate at 2 M ammonium sulfate was applied to determine capacity. Breakthrough was followed by agarose gel electrophoresis.

Bead diameter	Q_{10}
90 micron	0.5 mg/ml
30 micron	3.1 mg/ml
15 micron	8.3 mg/ml

Table 1b. Comparison of dynamic binding capacity at 10% breakthrough for three different samples with different sizes on Q Sepharose XL and Q Sepharose Fast Flow. Binding capacities are as indicated. BSA = bovine serum albumin, Ad5 = adenovirus type 5.

Molecule	Q_{10} Q Sepharose XL	Q_{10} Q Sepharose FF
BSA	130 mg/ml	110 mg/ml
Ad5	10^{13} vp/ml	1.6×10^{12} vp/ml
Plasmid	2.0 mg/ml	0.5 mg/ml

While some products enter Phase I with density-gradient purified material, Phases II and III require scaleable techniques. Process economy, required product quantities, and throughput make traditional density-gradient centrifugation steps a poor choice for large-scale purification.

Purification methods such as filtration and chromatography, which have been used with great success in the purification of proteins, are fully scaleable, economical, and compliant with cGMPs. However, any successful process design will have to take into account the unique size of viruses and plasmids.

General Considerations When Working at the Macromolecular Scale

Viruses and plasmids far exceed the molecular weights commonly seen for proteins. Molecular weights range from 7×10^6 daltons for a 5-kb plasmid, to 2×10^7 daltons for adenovirus, and up to 6×10^7 daltons for some of the larger enveloped viruses. Proteins are small by comparison, with molecular weights ranging from 0.005×10^5 to 0.8×10^5 daltons.

Hydrodynamic diameters of viral and plasmid entities are in the nanometer range, and the exact size is dependent on the salts present in the environment. Plasmid sizes are from 150 to 250 nm for the 5- to 10-kb size range, and viruses range from 20 to 1000 nm. Some viruses, such as rotaviruses, also carry a larger hydration shell. Here, hydrodynamic diameters can increase to almost double depending on the salt environment.[1] The high molecular weight and large hydrodynamic diameter have implications on the processing of such feed-streams. Two key factors are shear stress and resistance to mass transfer.

Today's bead-type chromatography media are not able to accommodate such large particles within their pore structure. Pore sizes of at least 15 µm

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are required to achieve good penetration by these molecules.[2] Such pore sizes would severely impair stability of current media. Thus traditional chromatography media only allow surface binding of plasmids and viruses for purification.

This is advantageous in some respects, as viruses and plasmids have a high resistance to mass transfer. Diffusion coefficients of viruses and plasmids differ from most proteins by a factor of 10- to 100-fold. The topography of a bead simply does not allow contact times sufficient for binding and elution. For surface binding only, this is of little consequence. Flow rates from 150 to 400 cm/h can be achieved without a severe loss in available capacity.

Aside from monoliths or membrane-based chromatography, traditional chromatography can overcome some of its limitations either by working with a reduced bead diameter, which increases surface area and hence capacity (Table 1a), or by increasing the surface area with long, flexible dextran chains for ligand presentation, as can be found on Q Sepharose™XL (Table 1b) and tentacle gels.[3–9]

Smaller beads are limiting in terms of pressure drop and price, which makes their use often impractical as a first step. Yet preclarified, tangential-flow (TFF) processed material is often sufficiently prepurified to be applied to bead diameters of 30 µm or less. The gain in capacity follows the gain in surface area, so that a threefold reduction in diameter implies an almost tenfold increase in capacity. This by far exceeds the increase in media cost for smaller beads, so that process economy is still improved.

Monolith and membrane adsorption techniques offer advantages in capacity, pressure-flow characteristics, and flow rates. There are, however, also some drawbacks. A recent publication reported large elution volumes as compared to the bed volume for an anion-exchange membrane, where, despite high capacity, only about a tenfold concentration could be achieved.[6] Others have reported recovery issues.[3] Also, monoliths cannot be packed on site but must be synthesized and packed by the manufacturer, with transport and validation consequences.[10] Few monoliths are currently available as products and they are still limited in scale.[10] Shear stress at the higher flow rates available with these novel types of adsorbers is high compared to chromatography techniques, so recoveries of gene therapy products may be impaired. Membrane adsorbers are limited to ion-exchange ligands at this point. It has been shown that for at least non-enveloped viruses, the use of chelating media and hydrophobic interaction chromatography media allows removal of empty capsids for adenovirus type 5 (Ad5).[11] Chelating chromatography has also been recently described for the purification of herpes simplex virus (HSV)-1.[12]

Critical Parameters in the Development of Scaleable Virus-Purification Processes

When designing a virus-purification process, planners should consider the effects of variations in the upstream components. Formation of viral aggregates can be dependent on both cell line and culture conditions. While some additives protect viruses from shear forces, they may interfere with purification. Addition of phenol red should be avoided if anion exchange is used. Phenol red can displace virus from columns, or mask and overlay viral peaks (Fig. 1). A well-defined starting material with set specifications (e.g., total

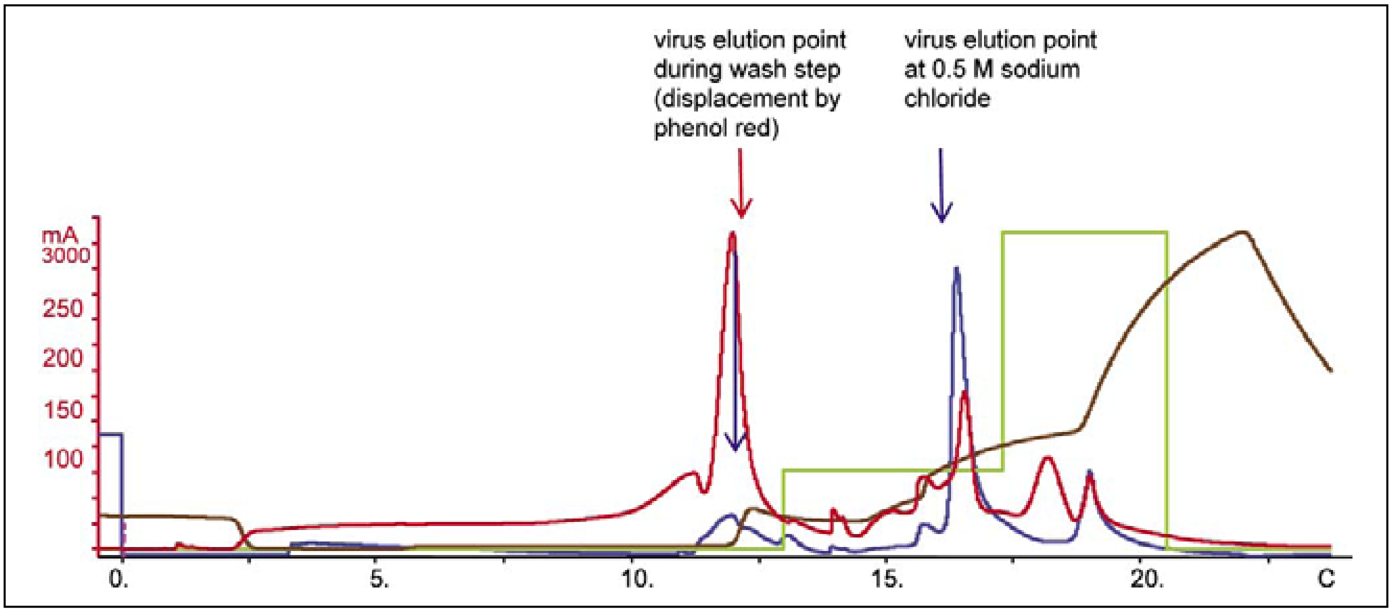

Figure 1. Overlay of 2 chromatograms showing the effect of 0.1% phenol red to a pre-cleared cell lysate containing a non-enveloped virus. Dynamic binding capacity at 5% breakthrough was 5 and 11 CV for the red and blue UV trace respectively. Red UV trace, A280 of the feed material adjusted to 0.1% phenol; blue UV trace, A280 of feed material without phenol red, green – gradient step protocol for elution of the virus; brown – conductivity. X-axis – column volumes. Y-axis – A280 in mAU. Column used HR5/50 Q Sepharose HP at 100 cm/h during sample application, elution at 75 cm/h. 10 CV applied for both experiments.

Table 2. The effect of operating shear rate on recovery of a non-enveloped 80 nm virus. MidGee hollow fibre cartridges were operated at an inlet pressure of 15 psi at 4000/sec, 6000/sec, and 8000/sec shear rates. Recoveries in percent are as indicated.

Shear s-1	Recovery Percentage
4000	30
6000	70
8000	75

protein mass, titer) is crucial for robust downstream processing of any virus.

Cell lysis due to shear may affect the amount of released genomic DNA and, hence, viscosity of the starting material when centrifugation is used for clarification. Interactions of the virus with DNA may be suppressed by adding low concentrations of magnesium or calcium (1–5 mM) or in the presence of high salt (>300–500 mM NaCl). Recoveries of virus may vary, especially for heparin-binding viruses, due to aggregation issues. Viruses that bind heparin are likely to bind DNA as well, which will compound aggregation issues. Some viruses are pleomorphic, and heterogeneity in size and density can cause problems during centrifugation.

Viruses were originally discovered by filtration.[13] Filtration is still the most prevalent method of virus removal.[14,15] Valuable information can be found in the literature on conditions that allow separation of virus from smaller molecules, as well as on an acceptable stability window. Before developing a virus-purification process, planners should set parameters for an acceptable pH range, salt concentration, and type, as well as temperature range, times, hold, and storage conditions. For example, reovirus was shown to lose infectivity when frozen in magnesium chloride.[16]

Viruses can aggregate above a certain titer or in a given salt or pH environment. Other critical parameters are shear rate and the highest possible titer. Interestingly, hollow fibers appear advantageous for the purification of viruses. They offer the benefit of low shear and, consequently, higher recoveries.[17] Tangential flow filtration should be optimized for shear rate (Table 2), inlet pressure, pump shear, pore size and pore-size distribution, load of the feed material, appropriate ratio of volume/m^2, and concentration factor.

For the design of a purification process, the intrinsic properties of the product — size, charge, hydrophobicity, and specific biological interactions — should be taken into consideration, with the goal of meeting final required purity specifications at maximal yield, with a minimal number of steps, and requiring as little conditioning or adjustment from technique to technique. Tasks should be assigned to each step (e.g., DNA removal, protease removal). If possible, cold room operations should be avoided. Optimization of each step, in addition to the overall process itself, is crucial for an economical, scaleable approach. Only process-scale media should be used, and step protocols should be developed prior to scale-up. It is critical to keep in mind that the total yield is the product of the yields from each individual step. Thus, the most economical approach is to reach the final purity target with the least number of steps. It is more economical to optimize the performance of each step than to introduce additional steps. However, neither the purity of the product nor the robustness of the process should ever be compromised.

Most viral-purification schemes exploit the large size of the virus either by ultrafiltration or group separation. Ion-exchange-based separations are also prevalent in scaleable purification procedures.[18–25] The charge behavior of a virus over a given pH range may be determined by electrophoretic titration curves.[26] The use of adsorptive techniques other than ion exchange has been described for Ad5, HSV, and adeno-associated virus (AAV).[11,12,27–29] Two-phase systems may be a scaleable alternative to traditional methods or steps.[30–34]

A basic flow scheme for virus-purification procedures is shown in Figure 2. It indicates some of the key parameters for purification of an active, infectious virus. Many viruses will bind to ion exchangers at low conductivity. Traditional ion exchangers may sometimes allow sample application at higher conductivity; for example, Ad5,

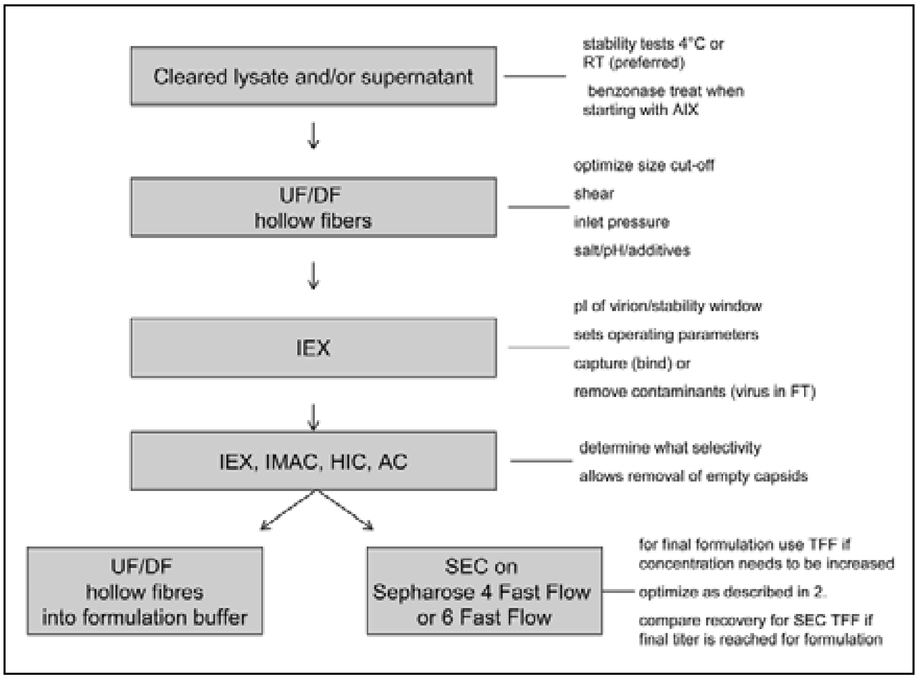

Figure 2. Generic flow scheme for scaleable, viral purification processes. AC – affinity chromatography; IEX – ion exchange; IMAC – immobilized metal affinity chromatography; HIC – hydrophobic interaction chromatography; SEC – size exclusion chromatography in group separation mode; UF/DF — ultrafiltration/diafiltration.

in which binding occurs up to 350 mM NaCl. Application at higher conductivity allows removal of additional contaminants in the flowthrough, but also lowers capacity. An inability to work at low salt concentrations with viruses that are prone to aggregation at low salt can be addressed by novel salt-tolerant ion exchangers.[35] Poliovirus requires the presence of at least 50 mM NaCl to prevent aggregation, but does not bind to traditional Q ligands under these conditions. The virus can be bound and concentrated to required titers on a custom-designed media (CDM) prototype with a ligand that allows binding even at high salt concentrations.[36]

Lid beads are another option to purify viruses that are difficult to recover once bound. Lid beads are designed to carry charges only within the bead pore structure so that the virus, by virtue of size, cannot bind. Most contaminants will be able to penetrate pores and bind to the ligands.[37]

Critical Parameters for the Development of Scaleable Plasmid Purification Processes

The final scale as well as final required purity should be determined before the purification system is designed. For example, a formulated plasmid therapeutic requires a smaller dosage than a naked plasmid therapeutic, and purity specifications will therefore differ.

Upstream parameters affect the ease of purification. Bacterial strain, copy number of the plasmid, size of the plasmid, time of harvest, growth media, and selective markers all affect downstream processing. The *E. coli* cellular lysate is rich in proteins at 55% and RNA at 20% dry weight. Lipids are found at 9% dry weight and genomic DNA at around 3%. Even in a best-case scenario, the amount of plasmid produced is only about 3% of the total biomass. Lysis methods used for plasmid purification are unique, exploiting the ability of small, circular DNA molecules to denature and renature and remain soluble compared to the bulk of the contaminants. Traditional methods and modifications thereof are still used today to lyse bacteria for plasmid purification.[38–44] Both alkaline lysis as well as heat-based lysis generate a starting material depleted of the bulk of protein, lipids and, when carefully optimized, genomic DNA. The remaining contaminants are RNA, residual proteins, genomic DNA, and endotoxins. The lysis step has been extensively studied.[44–50] Formulation and pH of the lysis buffer, duration of lysis steps, temperature of the lysis step, the ratio of cell paste to lysis buffer, the bacterial strain, and the size and sequence of the plasmid all affect the material generated. During lysis, the lysate becomes a non-Newtonian fluid of 20- to 40-cP viscosity. Plasmid DNA is denatured, extended, and in its most vulnerable state during this stage. Low-shear mixing at large scale is not a trivial process. This also holds true for neutralization.

Lysis is a key step in preclearance of contaminating genomic DNA and potential product loss due to irreversible denaturation. Optimization of this step for each plasmid is crucial.

Timely flocculate removal is critical as well. Time and shear mixing will release more contaminating genomic DNA and proteins back into the lysate.[51] More traditional approaches use bag or depth filters for flocculate removal. One very elegant approach describes the use of a two-phase system to remove the flocculate as well as to enrich and prepurify the plasmid DNA.[52]

The main contaminant to be removed after lysis is water. Tangential flow filtration is an ideal approach to enrich for plasmid DNA, removing proteins, smaller RNA molecules, and endotoxins in the mono- and dimeric forms.[53]

All adsorptive techniques suffer from the drawback that RNA and genomic DNA do compete for available capacity. Pretreatment with RNase can be beneficial but raises validation and safety issues. Therefore, approaches today use adsorptive techniques of unique selectivity or group separation, which allow capture of plasmid DNA.[54–56] Negative steps allowing capture of RNA and genomic DNA, but not plasmid DNA, have also been described.[37,57] Triple-helix chromatography allows selective binding of plasmids but can require a specific sequence and is offered only as a custom-made media.[54]

RNA is more hydrophobic than plasmid and binds more tightly to hydrophobic-interaction chromatography (HIC) and reverse-phase chromatography (RPC) media. Hydrophobic-interaction chromatography is, therefore, not a good choice to capture plasmid DNA, but may be used to remove RNA and genomic DNA. The main drawback is addition of ammonium sulfate to the lysate, which causes precipitation issues and reduces yield. Group

Table 3: Effect of different salts used on recovery and purity of a group separated plasmid applied at 50% capacity to an HR5_50 PlasmidSelect column at 2 M ammonium sulfate. ND=none detected.

Salt	Yield (%)	Endotoxin U/mg	gDNA µg/mg	RNA µg/mg	Protein µg/mg
2 M NaCl	87.0	1.3	0.14	ND	11
1.6 M $(NH_4)_2SO_4$	83.8	0.64	0.10	ND	11
2 M Mg SO_4	61.0	0.20	0.04	ND	<5
2 M $(NH_4)_2SO_4$	68.9	0.30	0.06	ND	<5
1.4 NaCl					
3 M $KC_2O_2H_3$	80.0	1.5	0.06	ND	10

separation, while allowing simultaneous buffer exchange and RNA removal, may be difficult to accomplish for processes operated at a very large scale, and nontraditional chromatography modes, such as Simulated Moving Bed, might be preferred. Alternative protocols on how anion exchangers alleviate the need for RNA treatment have been described as well.[58–61] Other approaches have described the use of gyrolites, immobilized metal affinity chromatography (IMAC), and affinity ligands.[62–63]

Hydrophobic-interaction chromatography has been applied as a second step in plasmid purification at ammonium sulfate concentrations from 1.8 to 3 M ammonium sulfate, depending on the hydrophobicity of the ligand, ligand density, and bead diameter. A thiolic, aromatic ligand offering a unique selectivity for supercoiled DNA has recently been introduced, which allows removal of the open circular isoforms to enrich for supercoiled DNA up to 99%.

When using adsorptive techniques, investigation of salt used during binding, washing, and elution is also crucial for optimal recovery. As an example, Table 3 shows a comparison of different salts and their impact on yield and purity of a plasmid product eluted from PlasmidSelect (a thiolic ligand), allowing selective removal of open circular DNA.

Even though the FDA has recently released a draft for chemistry, manufacturing, and controls (CMC) regulations for gene therapy products, required purity levels may very well be moving targets that are set by increasingly sophisticated processes.[64] These processes can achieve very high purity levels in as little as two or three steps by combining different selectivities for capture.

Tangential flow filtration may be used throughout the process, but size cutoffs need to be carefully evaluated. Plasmid size depends on the salt environment.[65] The buffer environment affects the selection of size cutoff. Effects of conductivity on plasmid loss in the permeate can be seen in Table 4.

Table 4. The effect of conductivity on plasmid loss in the permeate. All data were collected with a MidJet/MidGee system, operated at 4000/sec shear, TMP = 5 psi after concentration of tenfold. After 10x concentration plasmid loss was analyzed by agarose gel electrophoresis. Gels were stained with Vistra Green and scanned on a Typhoon fluorescent scanner. Image Master software was used for quantitation of plasmid DNA in retentate and permeate.

Feed	Conductivity of buffer (mS/cm)	Membrane size cut-off	Plasmid DNA-content in permeate	Plasmid DNA-content in retentate
PlasmidSelect purified plasmid DNA	245	750	6%	90%
	245	500	3%	97%
	245	300	n.a.	100%
	245	100	n.a.	100%
	145	750	3%	97%
	145	500	0%	100%
	145	300	0%	100%
	145	100	0%	100%

Outlook

When optimized carefully, current technologies allow the capture of plasmids and viruses to high purities that compare to the "gold standard" of ultracentrifugation. Chromatography and hollow-fiber techniques are sufficiently gentle to allow recovery of infectious virus, while supercoiled plasmid DNA and a combination of techniques using different selectivities (such as charge, hydrophobicity, and size) allow purification to very high purities and meet regulatory requirements. Both techniques are fully scalable for easy transfer to large-scale manufacturing. However, there is an increasing need for new chromatography media specifically developed for use in plasmid-DNA and virus-purification processes.

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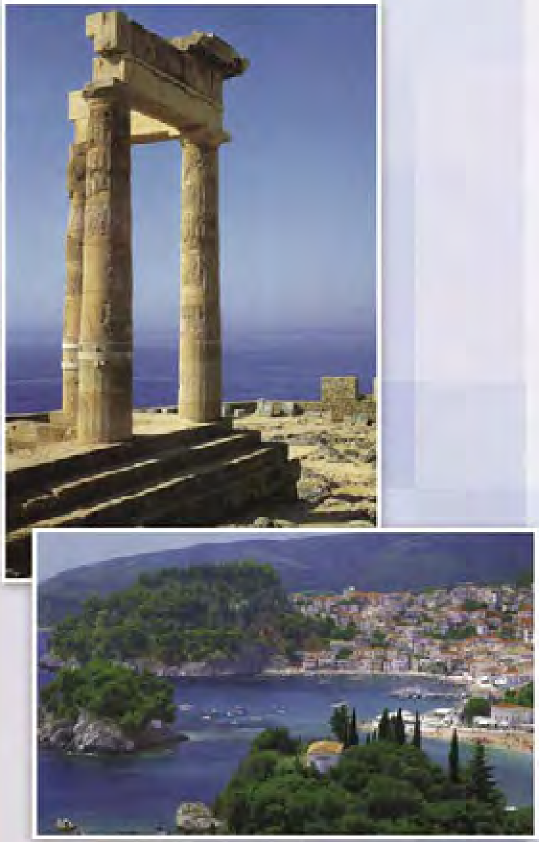

Highlights and Objectives of the Conference

1. Latest information on non-viral and viral vectors and expression systems

2. Highlights on recent development in cell engineering, cell culture technologies and protein production

3. Latest development in systems biology, RNA interference and stem cell/tissue engineering

4. Informal discussion with invited speakers and other delegates from both industry and academia

5. Latest information on reagents and equipment through vendors' exhibition and workshops

http: www.peace-conference.org

We invite you to submit an ABSTRACT
Deadline May 30th, 2005

7th PEACe
Knossos Royal Village Hotel
near the city of Heraklion.

WilBio

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WilBio – Asia/Pacific

Process Development and Production Issues for Cell Derived Products

2nd Annual Meeting

Four Seasons Singapore ◆ Republic of Singapore

September 4-6, 2005
Antibodies & Recombinant Proteins

September 7-9, 2005
Viral Vectors & Vaccines

TOPICS INCLUDE:

Antibodies & Recombinant Proteins:

- Product Characterization
- Viral Clearance
- Stability & Storage
- Assay Development
- Expression Enhancement
- Conjugation and Formulation
- Regulatory Issues
- Safety Testing
- Cell Line Development
- Purification

Viral Vectors & Vaccines:

- Vector Development
- Storage & Stability
- Viral Yield Optimization
- QA/QC Issues
- Assay & Media Development
- Reference Material Issues
- Raw Material Screening
- Product Characterization
- Downstream Processing

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