Procedure

# Experimental planning and mechanistic modeling workflow for the separation of full/empty AAV capsids with IEX

## Recommended procedure

A mechanistic chromatography modeling workflow typically requires a model calibration step, which includes system and column characterization, protein experiments, and parameter estimation (Fig 1).

This document gives guidance regarding biomolecule experiments to model adeno-associated virus (AAV) full/empty separation with ion exchange chromatography (IEX).

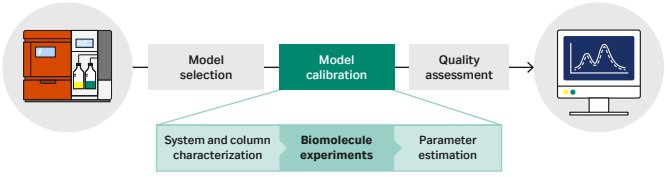


**Fig 1.** Outline of a mechanistic chromatography modeling workflow, with a focus on model calibration.

## I. AAV polishing with ion exchange chromatography

After upstream processing and a successful initial chromatography step, during which host cell proteins (HCP), DNA, etc. are removed, an AAV polishing step is needed for efficient separation of full and empty AAV capsids. IEX, especially anion exchange chromatography, is typically applied to exploit electrostatic differences between empty, partially filled, and full capsids for full capsid enrichment (Fig 2).

The separation can be induced in different ways.

- **Salt concentration:** the separation is realized by increasing the salt concentration in a gradient or step.
- **pH value:** the separation can be induced by changing the buffer pH.

Often, additives such as $MgCl_2$ or poloxamer are incorporated into the buffers to improve process performance.

## II. Experimental planning

The number of model parameters to be determined is dependent on model complexity, and the required precision is dependent on the model application. Modeling two elution influencing process parameters such as pH and additive concentration requires more model parameters than a simpler process with a fixed pH and additive concentration. Late-stage applications require more rigor than early-stage applications. Data quality is more important than data quantity.

In general, the experimental plan for ion exchange chromatography at a constant pH typically recommends five experiments to achieve suitable understanding of the capsid-adsorber interaction, kinetic limitations (adsorption and transport), and saturation behavior. If possible, all experiments should be performed with the UV absorbance monitored at 260 nm (DNA), 280 nm (protein) and 214 nm (peptide) wavelengths, corresponding to strong absorbance by DNA, aromatic amino acids, and peptide bonds, respectively. Fractions should be collected every 0.5 column volumes (CV), and samples of feed and flowthrough material should also be taken.

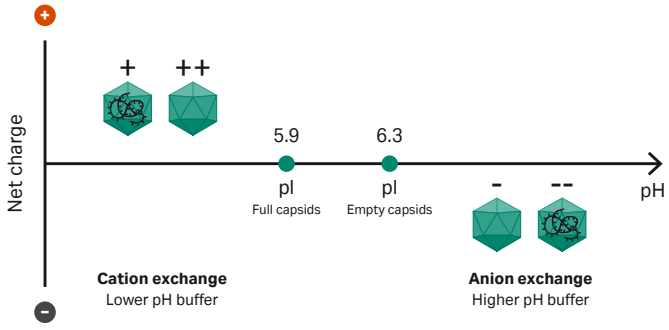


**Fig 2.** Net-charge, pH dependency of exemplary AAV full and empty species.



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These experiments are classified as

- **Three linear gradient elution (LGE) experiments** with a low load challenge (<$10^{12}$ viral particles (VP)/$mL_{adsorber}$ or at least a UV signal above 5 mAU)

  Performing experiments in the linear range of the adsorption isotherm enables studying thermodynamic behavior and can be used to define the equilibrium and charge parameters for both the steric mass action (SMA) and colloidal particle adsorption (CPA) binding models. These experiments can be performed at different flow rates if a flowrate dependency is important for model application.

- **One step elution experiment** with a low load challenge (<$10^{12}$ VP/$mL_{adsorber}$ or at least a UV signal above 5 mAU)

  To understand the kinetic limitations of the system, step elutions are used. If a difference between kinetics for each component is expected, the step elutions should be informed by the linear gradient elutions, in which the salt concentrations are chosen to desorb all relevant species (i.e., X% B to desorb empty capsids, Y% B to desorb partially filled capsids, and Z% B to desorb full capsids). These may be performed in the same experiment, with multiple steps. Alternatively, small step increments can be performed until complete elution is induced, i.e., 5% step increments up to 100% B. Good information about step elution experiment methods can be found in Hagner McWhirter (1).

- **One linear gradient elution experiment** at high load challenge (>$10^{12}$ VP/$mL_{adsorber}$):

  This experiment provides information about ligand shielding, which occurs due to steric hindrance or repulsive forces between the virus particles. This load challenge should be defined as in excess of the expected load challenge in the final process. Breakthrough is not required and is rarely achievable for AAV processes.

- **Optional: One injection of AAV** in non-binding conditions (high salt in mobile phase)

  This experiment can be used to determine the effective particle porosity for AAV capsids. Two injections at different residence times would be beneficial to estimate mass transfer resistance of the target molecule if this is likely to be significant (i.e., with packed bed adsorbers).

For complex processes, additional calibration experiments are required. The following experiments are recommended to account for additional dependencies, such as pH value or additives.

- **Buffer pH variation:** At least two LGE experiments should be performed, at the extremes of the pH range to be modeled. The pH range should be kept ± 0.5 pH units for simpler binding models such as SMA, whereas more sophisticated binding models such as CPA can often extend further than this, across multiple pH units.

- **Salt additives** (e.g., $MgCl_2$): As with pH modeling, additional LGE experiments are required to determine the correlation between adsorption isotherm parameters and additive concentration. For a simple system, LGE's are performed at two additional additive concentrations. As with pH, ranges should be chosen that are not excessive. Some additives may have more complex influence on adsorption behavior and so should instead be assessed with more additional LGE experiments at different additive concentrations.

Table 1 (*see* appendix) summarizes an experimental plan designed to optimize full/empty AAV separation at constant pH including $MgCl_2$ as additive. All parameters depend on the resin and AAV species and must be determined experimentally.

## III. Modeling workflow

To avoid the potential of overfitting the model, one should start with a simple model and only invoke complexity once the data demonstrate that complexity is required (i.e., complex peak shapes or poor fitting with a simple model). The equilibrium dispersive model is a good starting point, though some systems may require simulation of slow diffusion of AAV and would therefore be better described by a transport dispersive model.

One should begin by first fitting the most dominant species (often empty and full capsids). Offline and/or online analytics may be used for quantification of these species. If additional peaks are observed, these can be introduced as components, though best practice insists that these be eventually determined by an orthogonal assay if no analytical assay is available immediately, especially if these are considered critical impurities. Often, impurity classes are lumped together into a single pseudo-component or cluster. Aggregates can often be described as a single species as may partially filled capsids. If there is enough variability within a given cluster and this assumption fails to hold, lumping into a single cluster may be revisited and these clusters may be broken up into discrete species (i.e., dimers, trimers, and many different capsid variants).

Once the final model is calibrated to the precision required for the application, the model should be validated experimentally. The criteria for a successful validation are determined by model application and should ensure that the model is fit for the intended purpose. The validation experiment can, i.e., be performed at the desired process quality as derived from the *in silico* optimization. Other options are to choose process conditions at the edges of failure or to include experiments at different scales, depending on model application. The model error and its potential impact on product quality should be evaluated. The degree of certainty on the model parameters should also be considered.

## IV. Offline analytics

To determine the concentration and distribution of full and empty capsids and any other critical impurity, peak fractions should be collected and analyzed. Any quantitative analytics can be used for this, and the following assays have been used to good effect.

- Quantitative polymerase chain reaction (qPCR)
- Enzyme-linked immunosorbent assay (ELISA)
- Electron microscopy
- Analytical ultracentrifugation
- Gel electrophoresis
- Dynamic light scattering
- Functional assays
- Surface plasmon resonance (SPR)
- Anion exchange chromatography high-performance liquid chromatography (AEX HPLC)

### Reference

1. Hagner McWhirter Å, From cells to purified capsids: How to develop a scalable rAAV process. *Cell & Gene Therapy Insights* 2022; 8(3), 611–620. DOI: 10.18609/cgti.2022.094

**Table 1.** Exemplary experimental plan for salt dependent AAV full/empty separation.

| | Experiment | Purpose | Feed material | Comment | Analytics | Number of experiments |
|---|---|---|---|---|---|---|
| Characterization of fluid mechanics | Non-binding AAV | To determine effective column porosity for AAV and mass transport resistance | Loading material in a high salt sample matrix.<br>Running buffer should be the typical elution buffer. | Performed as a typical HETP experiment at pH set-point: small volume of tracer (1% to 2% of column volume), recommended flow rate for the adsorber used.<br>A single experiment will determine porosity, another experiment at a slower flowrate can determine diffusion rates. | UV (260 nm, 280 nm) | 1 or 2 |
| Characterization of adsorption behavior | Gradient elutions | To determine the ideal binding behavior of each component | Loading material in a low salt sample matrix, loaded to a low load challenge (< $10^{12}$ VP/mL$_{adsorber}$/UV signal should be above 5 mAU). Adsorber should be washed in equilibration buffer, and then undergo a linear gradient elution to a high salt buffer. | Three separate experiments to be performed, with the sole difference being the gradient length. For a typical packed bed adsorber, 10, 20, and 30 CV gradient lengths work well. Other adsorber formats may differ. | UV (260 nm, 280 nm), conductivity, pH<br>Offline analytics recommended | 3 |
| | Stepwise elution | To determine kinetic resistance of the system | Loading material in a low salt sample matrix, loaded to a low load challenge (< $10^{12}$ VP/mL$_{adsorber}$/UV signal should be above 5 mAU). Adsorber should be washed in equilibration buffer, and then undergo a stepwise gradient elution to a high salt buffer. | Only a single stepwise elution is typically required. If a vast difference in kinetics between components is expected, perform a stepwise elution at the conductivity that desorbs the components in question. | UV (260 nm, 280 nm), conductivity, pH | 1 |
| | Variant 1 (pH)<br>Variant 2 (additive) | To determine the influence of additive or pH on component adsorption | Loading material in a low salt sample matrix, loaded to a low load challenge (< $10^{12}$ VP/mL$_{adsorber}$/UV signal should be above 5 mAU). Adsorber should be washed in equilibration buffer, and then undergo a linear gradient elution to a high salt buffer. To be performed at the ranges studied (maximum and minimum pH or additive concentration). | For each desired parameter, at least two additional gradient elutions are required at the maxima and minima of the ranges. Some systems may have a more complex dependency and then would require at least one more gradient elution for each process parameter. | UV (260 nm, 280 nm), conductivity, pH<br>Offline analytics recommended | 2 (best case), 4 (worst case) |
| | High loaded | To determine the saturation behavior of target molecules and impurities | Loading material in a low salt sample matrix, loaded to a high load challenge. Adsorber should be washed in equilibration buffer, and then undergo a linear gradient elution to a high salt buffer. | The load challenge should be 80% of the DBC$_{10\%}$ value. If this is not possible, either follow adsorber manufacturer guidance or load adsorber to a typical value expected. | UV (260 nm, 280 nm), conductivity, pH<br>Offline analytics recommended | 1 |

## Recommendation

The standard demand on mechanistic modeling used for AAV polishing steps is to optimize the yield and purity of full/empty virus fractions. Therefore, the impact of salt gradient, buffer pH, and additive usage is investigated. The calibrated model can be used further for risk analysis or process scale-up.

### Find more information on modeling with the GoSilico™ Chromatography Modeling Software

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- General model calibration: CY25244
- Experimental planning for ion exchange chromatography: CY25250

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- Free web course: How to optimize AAV capsid separation
- Biacore™ SPR systems for titer analysis of adeno-associated virus
- Effective separation of full and empty adeno-associated virus capsids by anion exchange

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