CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

# Midstream unit operations: unsung heroes in AAV process development

Ratish Krishnan & Matthew Roach

Although the quest for a templated process to accelerate the race to commercialization for cell and gene therapies has largely remained elusive, we are starting to see scientific data from process development experts. One underappreciated area of process development is midstream unit operations, which consist of steps such as cell lysis, DNA digestion, and clarification. Here, an example of a fruitful collaboration between MilliporeSigma and Precision BioSciences to develop an integrated approach to an adeno-associated virus (AAV) harvest step will be presented, with key takeaways that can be implemented in everyday process development.

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This article will focus on cell lysis, nuclease digestion, and clarification steps. Lysis is commonly practiced in two main ways – physical or chemical – with each having its unique advantages and disadvantages. The main drawback of physical lysis is the high capital expenditure and possible thermal degradation of the virus. Chemical lysis using detergents such as TWEEN® and Triton® is the most common method. Despite the need to demonstrate the removal of these added detergents in the process, they remain popular since they are convenient, scalable, and can be cost-effective.

The lysis step is typically followed by nuclease digestion to eliminate residual DNA. Benzonase® endonuclease is often employed in the viral vector space and is cited in many journals and biological license applications. The enzyme breaks down virus-nucleic acid complexes, reduces the viscosity of process intermediates, and prevents fouling of downstream equipment. It is easily removed in subsequent

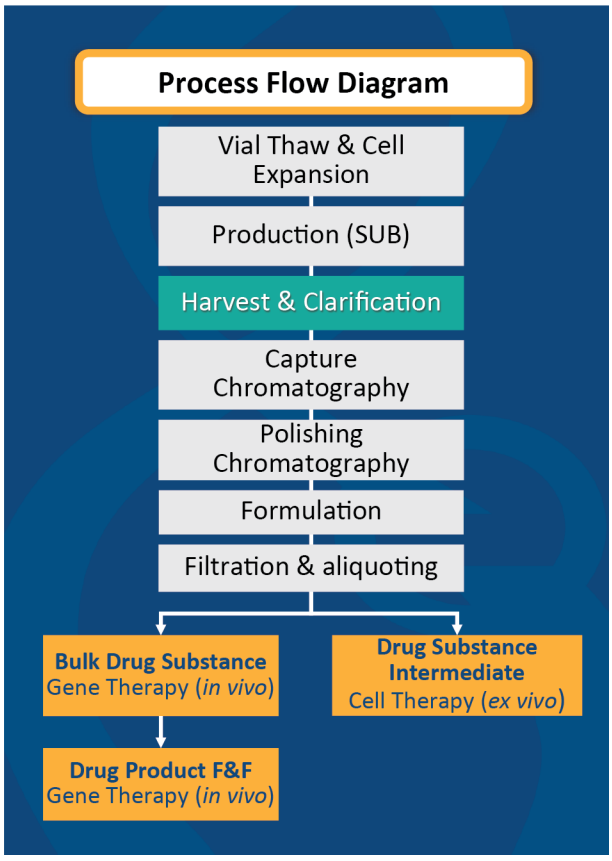
steps and has been used in several clinical trials and commercialized products, thereby assuring patient safety. One unit of the enzyme degrades approximately 37 µg of DNA in 30 minutes to as low as 3–8 base pairs, or less than 6 kDa. With the launch of Benzonase® endonuclease Safety Plus, the enzyme is completely animal-origin free, tracking back to upstream raw materials used in the fermentation process.

The workhorse of midstream is undoubtedly the clarification filters used in depth filtration. Filtration is typically carried out in two steps; primary filtration to remove large particles and secondary filtration for the removal of colloids and other sub-micron particles.

In this article, we will highlight data from a collaboration between MilliporeSigma and clinical-stage biotech company Precision BioSciences to optimize these midstream unit operations and improve yield.

**FIGURE 1**

Overview of AAV production process.




## INTRODUCING PRECISION BIOSCIENCES

Precision BioSciences is a clinical-stage biotech with pipelines for both allogeneic CAR T and *in vivo* gene editing, based in Durham, North Carolina. Their gene-editing technology, ARCUS®, is based on a naturally occurring genome-editing enzyme called a homing endonuclease. Having observed some of the limitations of other gene-editing technologies, Precision BioSciences looked to nature for better specificity, accuracy, and versatility, starting with the natural enzyme I-CreI from algae and reprogramming it to target new genetic sites.

With the ARCUS® technology, Precision BioSciences set out to target two main areas, the first being permanent correction of genetic disease with *in vivo* editing, and the second being the optimization of allogeneic CAR T cells for deep and durable responses to cancer. The company is currently in clinical trials for multiple cell therapy programs targeted towards a number of lymphomas. It also has a robust *in vivo* gene editing pipeline.

Both *in vivo* gene editing and CAR T cell applications utilize AAV, so Precision BioSciences has developed internal capabilities and knowledge around AAV production and the eventual manufacturing process.

## DEVELOPING A CLARIFICATION PROCESS

AAV is unique when compared to traditional biologics. Production processes typically require the addition of plasmid or virus to deliver the genes required for AAV production, and the removal of process-related impurities is even more critical as the ratio of impurities to product is higher than traditional biologics such as monoclonal antibodies.

With this in mind, Precision BioSciences set out to develop its harvesting clarification process with three primary goals. First, to ensure a high viral genome recovery throughout the harvest process by reducing host cell debris and contaminants. Second, to ensure it is a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platform block that can be used for many different AAV serotypes, to target different tissues. Lastly, to lower the cost of goods and complexity of the process, specifically by reducing the total filtration area required for a given batch and the number of types of filters needed for the filtration. Figure 1 gives an overview of the harvesting process, including lysis with endonuclease treatment and depth filtration.

Here, we will focus on the results from the development of Precision's platform depth filtration step for AAV clarification. TWEEN® 20 was used to lyse cells and release intracellular AAV, followed by endonuclease treatment to reduce contaminating host cell DNA, depth filtration, and 0.2-µm filtration to remove additional host cell debris.

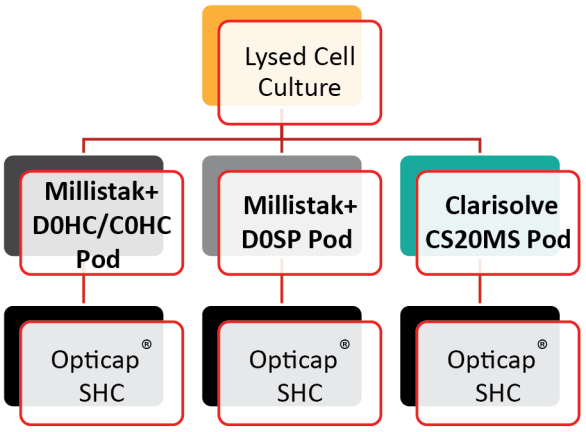
## DEPTH FILTRATION SCREENING STUDY

Based on the data mining summary provided by MilliporeSigma, and with the support of Technical and Scientific Solutions team, a limited number of depth filters for filtration of lysed cell culture were screened with Precision BioSciences. One of the benefits for Precision BioSciences in their collaboration with MilliporeSigma was the ability to rely on MilliporeSigma's expertise about their products. For example, MilliporeSigma scientists were able to rule out filters that were unlikely to work for the process based on their prior experience with AAV production.

We took lysed cell culture through three different depth filtration trains, one D0HC and C0HC, one with D0SP, and one with CS20MS – all followed by the Opticap® SHC filter from MilliporeSigma (Figure 2).

**FIGURE 2**

**Screening of Millistak+® HC Pod Depth Filters, Millistak+® HC Pro Pod Depth Filter, and Clarisolve® Depth Filter.**




D0HC *et al.* level is "depth filtration".

The Millistak+® HC Pro Depth Filter utilizing the D0SP media series was selected for further testing and scale-up as it achieved the highest throughput in the depth filtration screening study (Table 1). Additionally, it provided high viral genome and viral particle recovery and was capable of producing a stable clarified lysate, monitored via turbidity measurements. We saw viral genome recovery and viral particle recovery above 100% in this experiment. This level of variance was within the normal observed range for both assays at this time of analytical development. Interestingly, the Millistak+® D0SP pod depth filter was able to provide throughput much greater than the Clarisolve® 20 and Millistak+® D0HC/C0HC filtration trains, although all performed well enough to scale. Due to time constraints, further process development was performed with just the Millistak+® D0SP filtration train.

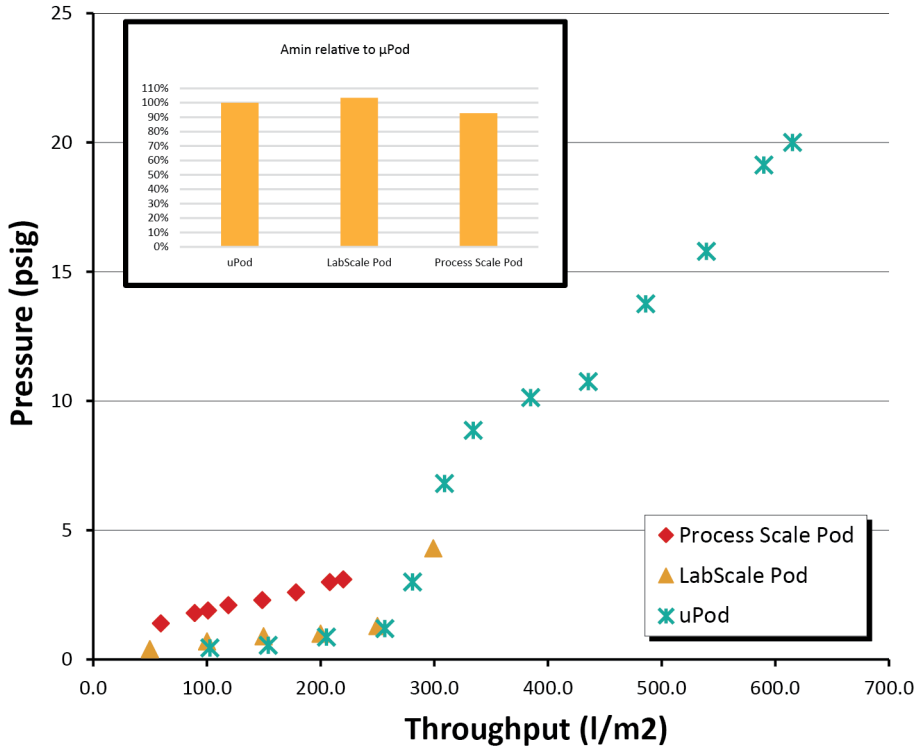
**TABLE 1**

**Key results from the depth filtration screening study.**

| Filter | Throughput (L/m²) | Viral genome recovery (%) | Viral particle recovery (%) | Stability of the clarified lysate |
|---|---|---|---|---|
| Millistak+® D0HC/C0HC Pod depth filter | 179 | 111% | 120% | 3 days |
| Millistak+® D0SP Pod depth filter | 444 | 100% | 109% | 7 days |
| Clarisolve® CS20MS Pod depth filter | 218 | 101% | 124% | 3 days |

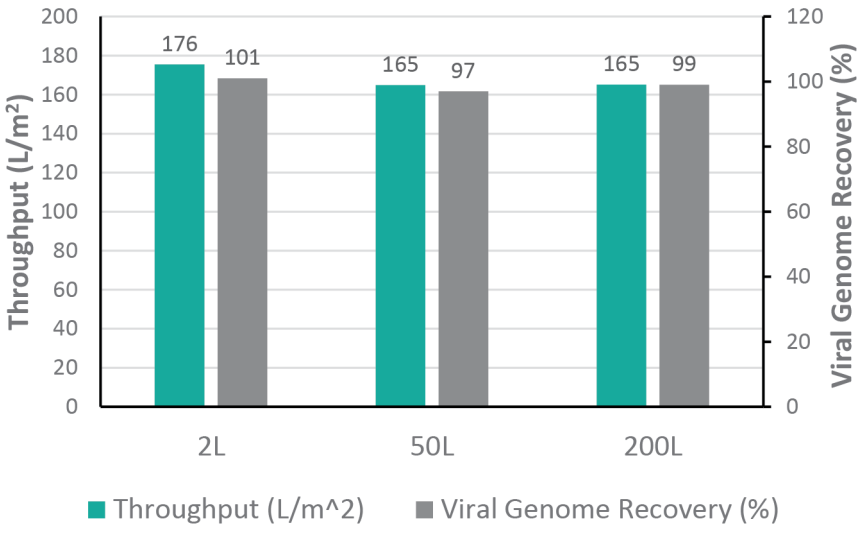
## FIGURE 3

Throughput with increasing pressure for three pods from MilliporeSigma.




## FIGURE 4

Throughput and recovery results across scale.





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## SCALE-UP BASED ON SELECTION FROM DEPTH FILTER SCREENING

MilliporeSigma has a range of depth filtration offerings across various process scales. Figure 3 shows pressure as throughput increases across each of the pod depth filter offerings. This case study with AAV gave Precision BioSciences confidence that the process could be effectively scaled up. Additionally, the pilot and process pod depth filtration system minimize bioburden risk through a single-use flow path and the ability to sterilize the pod with an autoclave.

After a lead depth filter was chosen, the Millistak+® D0SP filtration train was scaled up to 2L, 50L, and 200L, while monitoring the throughput and viral genome recovery. At a near-constant throughput, very similar viral genome recoveries were achieved across scale-up, indicating that the case study conducted earlier by MilliporeSigma was accurate (Figure 4).

One of the original goals was to ensure that the selected depth filtration train was capable of clarifying feed streams effectively, even when challenged with a new AAV serotype. After selecting a lead depth filter, the Millistak+® D0SP and OptiCap® SHC (sterile, high capacity) filtration train was advanced with 2-liter bench-scale runs of four different AAV serotypes carrying the same transgene, to confirm that the clarification train worked as a platform. Figure 5 details the throughput and viral genome recovery for each of these filtrations. Notably, even when the filtration train was challenged with a higher throughput, all serotypes achieved high viral genome recovery utilizing the Millistak+® D0SP and Opticap® SHC filtration train, with viral genome recovery at 84–96%.

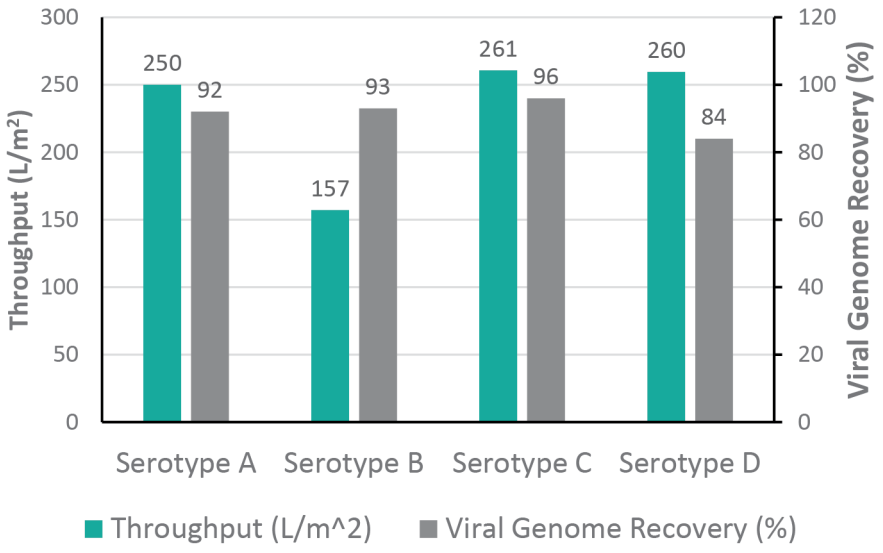
## CONCLUSIONS

These data show how collaboration between process and product experts can help solve problems in bioprocessing. Key takeaway messages from the study include:

- Focus on DOE approaches for optimization: it is very important to screen a variety of filters to data-mine the best fit for your process;
- All decisions should be based on the foundation of data;

**FIGURE 5**

**Bench-scale throughput and recovery results across serotypes.**



- Remember that adjacent unit operations impact clarification;
- Begin with the end in mind: think about scale-up from the start;
- A platform approach is possible: unit operations could be templated for other programs with additional optimization work;
- Moving from two-stage to single-stage filtration lowers the cost of goods and simplifies the process.

# ASK THE AUTHORS

**Ratish Krishnan**
Senior Strategy Consultant, Novel Modalities BioProcessing Group, MilliporeSigma

**Matthew Roach**
AAV Process Development Team Leader, Precision BioSciences

Authors Ratish Krishnan (MilliporeSigma) and Matthew Roach (Precision BioSciences) answer your questions about midstream unit operations.

**Q** When screening depth filters, which is better: high yield or high throughput?

**RK:** I would say the selection of a depth filter is based on balancing a lot of factors. Oftentimes we look at yield first, but if yields of the filter you've evaluated are comparable, sometimes the decision is based on the throughput. Other considerations may also include impurities, if applicable, like host cell protein and DNA. So I would say it's not a straightforward answer, but completely dependent on your feed, scaling requirements, and process expectations after you analyze the data.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**Q** And what is the difference between Millistak+® D0SP and Clarisolve® C0SP filters? How are they different from Clarisolve® filters?

**RK:** The Millistak+® HC Pro high-capacity synthetic media are a family of synthetic depth filters, providing cleaner and more consistent depth filtration media compared with usually used diatomaceous earth and cellulose esters. Multiple media grades are available and are used in primary and secondary clarification as well as downstream filtration. The Millistak+® D0SP is a four-layered depth filter media, which also includes an upstream non-woven layer to improve the filtration capacity, primarily used in direct harvest applications. Clarisolve® C0SP is also a four-layered depth filter media and it may be used either for direct harvest or secondary clarification purposes. Both have the benefit of synthetic materials, formulation design, and disposable parts, and can clarify anywhere from 5 liters to 22,000 liters.

Clarisolve® technology was developed to address the challenge of high cell density feed streams, where pre-treatment methodologies like PDADMAC or similar flocculating agents are typically used. It has a gradient density structure and is designed for the particle size distribution of pre-treated feed streams. Clarisolve® filters with the designation -20, -40, and -60 refer to the particle size distribution after treatment with flocculants or acid precipitation. This enables a single-stage clarification of the pre-treated feeds, and thereby reduces your footprint and eliminates the need for a secondary stage of clarification.

**Q** What is the advantage of using the step filters as opposed to using other depth filters, such as glass fiber filters?

**RK:** Glass fiber filters have very fast flow rates, high loading capacity, thermal tolerance, and particulate reduction. What we've seen, when you look back at monoclonal antibody bioprocessing, is that depth filters have become the staple in clarification technologies. They have better scalability, and better removal of residual impurities and sediments. In theory, you could use glass fiber filters, but we've seen depth filters being preferred in clarification operations.

**Q** Do you have any further data that confirmed the selected filtration train?

**MR:** Absolutely, yes. We have confirmed this with larger scales and across many different serotypes and transgene combinations, and even in just routine research production, we've seen this be a success. So it's definitely become a platform step for us.

**Q** How do you explain a greater than 100% recovery for every filter used in the filter study?

**MR:** With any assay, you're going to have inherent variability, even on the same plate. For an assay like ddPCR, we might see a 1–8% CV value. The viral particle titer is measured by ELISA, which has a smaller dynamic range to work with and so is a lot more variable.

**Q** How did you measure the stability of the clarified lysate?

**MR:** The data we showed here was gained through turbidity measurement of the clarified lysate. Since then, we have taken that through to capture chromatography as well, and soon we plan to take this to a final indication of stability through chromatography, and then testing out infectivity and potency.

**Q** Did Precision BioSciences evaluate alternative filtration options?

**MR:** We did, although we haven't included that data here. We're confident that this train was the best that we tested out. I think the recovery data speaks for itself and our host cell protein clearance and host cell DNA was more than sufficient as well.

**Q** You mentioned that your lysis is based on TWEEN® 20. Can you share the concentration, temperature, and time of incubation? Were the experiments performed on fresh or thawed harvest?

**MR:** There was a final concentration of 1%. The temperature was 36–37°C, and the time of incubation was 30 minutes for lysis. The experiments were performed on fresh harvest material on the day of harvest.

**Q** Were there any impacts of Millistak+® D0HC or Clarisolve® C0SP filters on AAV potency or host cell proteins?

**MR:** No. When we've compared the final material across the train (not at the step for stability) we have seen no difference in potency or final host cell protein from the processes.

**Q** What detergents were used for cellular lysis? And were the matrix effects on the analytics taken into account?

**MR:** We used TWEEN® 20 and the matrix effects were something we put a lot of effort into investigating. Our ddPCR team here at Precision BioSciences have worked


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tirelessly and tested a ton of conditions to ensure our assay is telling us exactly what we think it is – which is critical to making sure that the data we're showing here are accurate.

**Q** Have you been able to optimize the use of Benzonase® endonuclease Safety Plus in your process?

**MR:** Yes, absolutely. Ratish and his colleagues at MilliporeSigma were able to help out with that and have pre-set design of experiments that you can go through for it. Making use of those resources has saved us a lot on cost of goods and also made us confident that we are reducing host cell DNA at that step as effectively as possible.

**RK:** It's also very important to look at the unit operations connected to your depth filtration. Whether you're using Benzonase® endonuclease before or after, it's important to analyze the impact of each unit operation. The need for optimization is there, but it also depends on the analytics you trust at that point, so it often gets overlooked. If you optimize the Benzonase® endonuclease and then you do your clarification then you could get better surface area from your depth filters – it definitely has a knock-on effect.

**Q** Could you talk about the need for salt addition after cell lysis or Benzonase® endonuclease treatment and its impact on the filter performance?

**MR:** It's typically recommended to add salt for activation of Benzonase® endonuclease to stop that reaction. But we've actually seen the addition of salt helps us with recovery, and getting through that filtration process. So we've kept it in our process for both inactivation of Benzonase® endonuclease and ease of clarification.

**Q** For the depth filter trains, are you priming before loading the crude harvest?

**MR:** We're just doing a water flush – we're not doing a buffer flush. We have experimented a little bit with that but we haven't seen a large difference in recovery or stability.

**Q** Have you investigated implementing the addition of Benzonase endonuclease after clarification?

**MR:** That's something that we haven't studied but I think it's an interesting concept and maybe something that would pair really well with a TFF step after clarification.

**RK:** It really depends on how you want the train to be designed – there are lots of options. We have customers who use Benzonase® endonuclease before or after clarification, or even both.

**Q** How much clearance of host cell DNA does the Benzonase® endonuclease provide? And do the subsequent chromatography steps help to reduce any residual hcDNA?

**MR:** I can't speak to exact values, but we do see a large host-cell DNA reduction there. We also see a large reduction across our chromatography steps as well. I think this will be really dependent on your production processes – whether you're using a triple transfection system versus the baculovirus system, or other viral means of introducing the genes to produce AAV. Plus, what cell densities you're operating with.

**Q** Were there differences in the Opticap® SHC filter throughput based on the depth filter used?

**MR:** We have seen a difference in Opticap® SHC filter throughput depending on the filter, and it really seemed to correlate with where the cut-off is for the lower filter. For example, the Millistak+® D0SP filter has a higher final cut-off than Clarisolve® C0SP filter. The closer the cut-off of the depth filter is to 0.2 microns, the higher the throughput for the Opticap® filtration. We've been okay with our current train, but if you're trying to minimize the filtration area of the Opticap® and want to increase the number of depth filters on the other side prior to that, you can do that. It just depends on the facility design and process design.

**Q** What are your thoughts on investigating osmolarity, especially during the lysis step?

**MR:** It's a really interesting concept and there have been a couple of recent presentations by groups looking at that. It's something that we've measured, but it's not something that we've delved into too deeply yet.

**Q** How important is precise control of temperature and pH during the Benzonase® endonuclease step?

**RK:** Very important! I'd recommend taking a close look at the datasheet for Benzonase® endonuclease, because it's got a specific window of operation in terms of pH, temperature, and salt concentration. So it's important to make sure that your process is compatible with that. For a lot of customers adding Benzonase® endonuclease to a


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bioreactor is much easier because you've got the temperature control right, versus using it as a standalone operation, where you might have to do your Benzonase® endonuclease step at room temperature instead.

## Q How would different types of depth filters affect the overall lysate stability?

**RK:** I don't think it's a major concern. Most of the depth filters we've used don't appear to affect the lysate stability. Obviously, it's feed-dependent, but given AAV is produced in HEK cells or SF9, there are probably much lower cell densities than is typical and I don't see them affecting the stability overall. Common practice is an overnight hold at around 4°C or no hold in continuous processing.

**MR:** We haven't seen any effect on lysate stability either. But regardless, you definitely want to measure that and be sure that you're not missing out on something that may surprise you later.

### BIOGRAPHIES

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Ratish Krishnan is a Senior Strategy Consultant in the Novel Modalities BioProcessing group for the Americas at MilliporeSigma. He is passionate about providing solutions to bring treatments to market. A Process Development Scientist by background, he has over 13 years of experience in vaccine, monoclonal antibodies and viral vector modalities from pre-clinical to late-stage process characterization, validation and commercialization activities such as BLA authoring. As a Biochemical engineer, he holds a Master's degree in biotechnology from the Pennsylvania State University. Ratish has managed process development teams at Novartis and Pfizer prior to his current role where he serves as global subject matter expert for viral vector manufacturing and provides strategic guidance to internal stakeholders and key customers. He is active in his thought leadership activities at scientific conferences, technical webinars and key authorship contributions in peer-reviewed articles and white papers.

**Matthew Roach**
**AAV Process Development Team Leader, Precision BioSciences**

Matt leads the AAV Process Development group at Precision BioSciences, which is focused on designing and implementing new strategies for the production and purification of adeno-associated virus. Matt completed his Bachelor's degree in Biological Sciences at North Carolina State University and his Master's degree in Microbiology and Cell Science at the University of Florida. Prior to Precision, Matt spent time at Pfizer working on the purification of AAV and the Biomanufacturing Training and Education Center training industry professionals on downstream bioprocessing operations.

## AUTHORSHIP & CONFLICT OF INTEREST

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